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13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)*

The lymphatic system serves as the primary route for the metastasis of breast cancer, and the extent of lymph node involvement is a key prognostic factor for the outcome of the disease. Whereas the significance of angiogenesis for tumor progression has been well documented, the ability of tumor cells to induce the growth of lymphatic vessels, (lymphangiogenesis) and the presence of intratumoral lymphatic vessels have been questioned. We demonstrate occurrence of intratumoral lymphangiogenesis within human breast cancers after orthotopic transplantation onto nude mice, using a novel marker for lymphatic endothelium, LYVE-1. We show using green fluorescent protein-tagging that breast tumor cells which overexpress vascular endothelial growth factor-C (VEGF-C), display a potent increase in intratumoral lymphangiogenesis, and significantly enhanced metastasis to both regional lymph nodes and lungs. Furthermore we have found that the degree of tumor lymphangiogenesis is highly correlated with the extent of both lymph node and lung metastases. These results establish the occurrence and biological significance of intratumoral lymphangiogenesis in breast cancer and identify VEGF-C as a molecular link between tumor lymphangiogenesis and metastasis.

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Table of Contents

1. INTRODUCTION

The metastatic spread of tumor cells is a major cause of death in cancer patients. Breast cancer cells, like the majority of human carcinomas, metastasize preferentially via the lymphatic system. In fact, the spread of breast cancer cells via lymphatic vessels to the regional lymph nodes is one of the most important indicators of tumor aggressiveness, and the extent of lymph node involvement is a major determinant for the staging and the prognosis of the disease. It remained unknown, however, whether tumor-associated lymphatic vessels play an active role in tumor dissemination. The views regarding the presence of lymphatic vessels in tumors are conflicting and it remained unclear whether tumors stimulate the formation of new lymphatic vessels, i.e. lymphangiogenesis. The major objective of this study was to assess the significance of lymphangiogenesis for breast cancer progression. We have tested and ultimately proved the hypothesis that induction of tumor lymphangiogenesis promotes breast cancer metastasis.

2. BODY: RESEARCH ACCOMPLISHMENTS

Specific Aim I. To examine the extent of lymphangiogenesis in breast cancer and its relationship to tumor metastases.

Our limited insight into the status of tumor-associated lymphatic vessels has largely been due to the lack of the tools that allow specific visualization of the lymphatic vessels. We have employed an antibody to LYVE-1, the hyaluronan receptor specific for the lymphatic endothelium to visualize lymphatic vessels in biopses of human breast cancers, and we were able to visualize lymphatic vessels within and surrounding human breast cancers. Intratumoral lymphatic vessels were detected only within the metastatic tumors, however, we could not at this point establish the correlation of lymphatic vessel density and distribution with tumor metastasis, due to the insufficient number of samples that were made available for the study. We will further extend this study to the larger number of specimens that have been made available to us. In conclusin, we could confirm our prediciton that lymphatic vessel are found within metastatic human breast cancers.

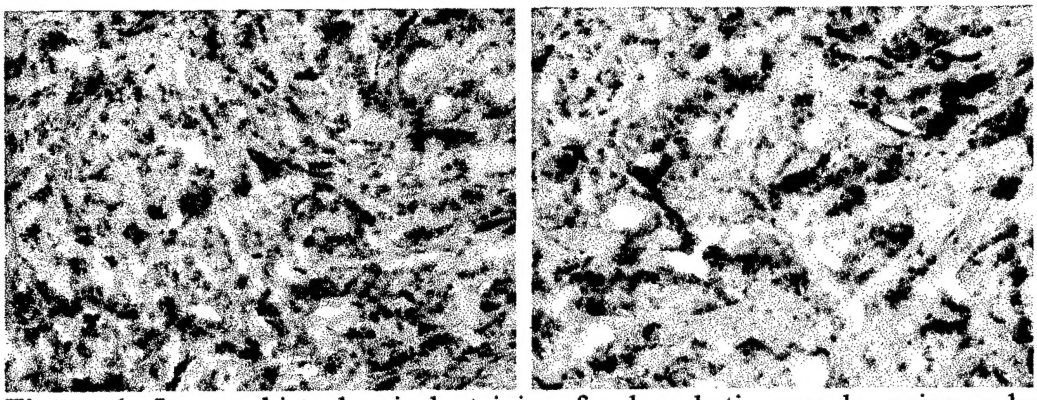

Figure 1. Immunohistochemical staining for lymphatic vessels, using a lymphatic-specific marker LYVE-1, of human breast cancer tissue. Note presence of many lymphatic vessels within a tumor at low (left pannel) and high (right pannel) magnification.

Specific Aim II. To assess whether induction of lymphangiogenesis promotes the metastatic capability of breast cancer cells.

Infiltration of lymphatic vessels by tumor cells has been found in vast number of experimental and human tumors, and the significance of lymphatics as a conduit for tumor cell dissemination has been well recognized. However, the presence and potential function of lymphatic vessels in tumors have remained controversial, due to the lack of molecular markers that reliably distinguish the lymphatic vasculature from blood vessels. Whereas some early studies reported intratumoral lymphatic vessels in certain types of cancer including breast cancer, this has been interpreted mainly as cooption of pre-existing lymphatic vessels by invasive tumor cells, and it has been proposed that lymphatic vessels are absent from most tumors. Moreover, evidence for an active molecular interaction of tumor cells with lymphatic vessels is still lacking, and it has

remained unclear whether tumors stimulate lymphangiogenesis and what, if any, might be the significance of this process for tumor progression.

The results of our studies that addressed the significance of lymphangiogenesis for breast cancer metastasist have been published [1], and are summarized herewith. We revealed the occurrence of pronounced lymphangiogenesis within human metastatic breast cancers in nude mice, making use of a recently identified specific marker for lymphatic vessels, LYVE-1. LYVE-1 expression is absent from blood vessels in human tumors, whereas expression of lymphatic marker VEGFR-3, while restricted to lymphatic endothelium during late stages of embryonic development and in normal adult tissues, has recently also been detected on blood vasculature in breast cancer. Our own results demonstrate expression of LYVE-1 in tumor-associated vessels that exhibit traditional characteristics of lymphatic vessels such as lack of a continuous basement membrane and very weak expression of the endothelial cell marker CD31. The expression pattern of LYVE-1 is in accordance with its presumed physiological function as a receptor involved in the transport of interstitial hyaluronan to the lymph nodes.

Our results demonstrate the presence of lymphatic proliferation within the peripheral areas of MDA-MB-435/GFP control tumors, suggesting the production of lymphangiogenic factors in this metastatic tumor. Overexpression of VEGF-C resulted in enlargement of peritumoral lymphatic vessels and in strikingly increased intratumoral lymphangiogenesis, identifying VEGF-C as a potent tumor lymphangiogenesis factor. We frequently observed proliferation of lymphatic endothelial cells within VEGF-C overexpressing tumors, and anatomically well defined lymphatic vessels were detected throughout the tumors at a much higher density than in control tumors. The greater depth of lymphatic vessel invasion into VEGF-C overexpressing tumors suggests a pronounced effect of VEGF-C on lymphatic endothelial cell migration in vivo. Whereas the overall lymphatic vessel density was fairly consistent within each tumor group, we found considerable regional heterogeneity within individual tumors, in particular within tumors overexpressing VEGF-C. This may reflect local variations of the tumor microenvironment such as differences in extracellular matrix composition and/or mechanical forces. Because of the unique structural and functional integration of lymphatic vessels with the interstitium, critical for the lymphatic function, a permissive extracellular microenvironment is likely to be also critical for lymphangiogenesis.

A large number of studies have failed to identify functional lymphatics within tumors, leading to the concept that lymphangiogenesis may not play a major role in tumor progression to the metastatic phenotype. However, the absence of detectable perfusion of tumor lymphatic vessels does not necessarily imply absence of anatomically distinguishable lymphatic vessels from tumors. Moreover, whereas the functional state of tumor-associated lymphatic vessels with respect to the efficient transport of fluids and macromolecules is of great importance for overall tumor physiology and drug delivery, it may not be critical for tumor dissemination. Indeed, the formation of an intratumoral lymphatic network, whether functional in fluid transport or not, may promote metastatic tumor spread by creating increased opportunities for metastatic tumor cells to leave the primary tumor site.

This concept is strongly supported by our findings that the increased density of lymphatic vessels within VEGF-C overexpressing breast cancers was associated with a significantly, more than 60% higher incidence of metastases in regional lymph nodes.

Therefore, our findings provide a mechanistic explanation for the recently reported correlation of VEGF-C expression in the primary tumors with high incidence of lymph node metastases in breast, colorectal, gastric, thyroid, lung, and prostate cancers. Moreover, a recent study found that VEGF-C expression was only detectable in node-positive breast cancers, whereas expression of VEGF was detected in both node-positive and node-negative tumors. The correlation between VEGF-C expression and lymphangiogenesis in these tumors remains to be established. However, while VEGF-C may promote the incidence of lymphatic metastases simply by increasing the number of lymphatic vessels in the vicinity of tumor cells, it is tempting to speculate that activation of lymphatics by VEGF-C or related factors promotes molecular interactions of tumor cells with lymphatic endothelial cells, facilitating tumor cell entry into the lymphatics. To investigate the molecular mechanisms of tumor cell interactions with the lymphatic endothelium, we have developed a novel technique for isolation of microvascular lymphatic endothelial cells and have extensively characterized those cells. The results of these study are now in press [2].

VEGF-C overexpression in primary tumors resulted in a significant increase of lung metastases. This was most likely not due to accelerated growth rates of metastatic MDA-MB-435/GFP-VEGF-C cells in the lung, since VEGF-C overexpression did not confer a growth advantage to tumor cells at either the primary tumor site or in lymph nodes. Furthermore, we did not observe an increase in tumor angiogenesis in VEGF-C expressing tumors, making it unlikely that an increased rate of dissemination via blood vessels accounted for the increase in lung metastases. Importantly, the extent of intratumoral lymphatic vessel density was highly correlated with the extent of lung metastases, implying an important role of lymphatic vessels for the metastatic tumor spread to distant sites. From the lymph node, tumor dissemination can occur by efferent lymphatics or through lymphatic-venous communications within the nodes, and subsequently via the blood stream. Although the exact pathway of breast cancer metastases to the lungs remains to be determined, our results suggest an increased incidence of lung colonization by tumor cells leaving the primary tumor site via the lymphatics. Since the lymphatic system is a low pressure and low flow system that is physiologically optimally adapted for the transport of cells, the preferential metastasis via lymphatics, due to expression of lymphangiogenic factor(s), might promote survival of disseminating tumor cells and thus increase their metastatic efficiency.

Our findings identify, for the first time, an active, growth factor mediated interaction between tumor cells and tumor-associated lymphatic vessels with important implications for the formation of regional and distant metastases. An improved understanding of the molecular mechanisms that control metastatic tumor spread may enable early recognition of the metastatic potential of primary cancers and the development of new therapeutic strategies for limiting cancer spread.

3. KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated

- occurence of lymphangiogenesis in experimental and human breast cancers
- significance of tumor lymphangiogenesis for metastasis in the mouse tumor model
- that VEGF-C is a tumor lymphangiogenesis factor
- significance of VEGF-C for tumor metastasis

4. REPORTABLE OUTCOMES

Manuscripts:

1. Skobe M., Hawighorst T., Jackson D., Prevo R., Janes L., Velasco P., Riccardi L., Alitalo, K., Claffey K., Detmar M. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature Med.* 2001; 7:192-198

2. Swartz M.A. & Skobe M. Lymphatic function, lymphangiogenesis and cancer metastasis. *Microscopy Res.* 2001; 55:92-99. Review.

3. Cassella M. and Skobe M. Activation of lymphatic vessels in cancer. *Ann NY Acad Sci* 2002; (in press)

4. Podgrabinska S., Braun P., Velasco P., Kloos B., Pepper M.S., Jackson D.G. and Skobe M. Molecular characterization of lymphatic endothelial cells. *Proc. Natl. Acad. Sci. USA.* 2002; (in press)

Presentations:

1.	09/2002	*"Angiogenesis: Molecular Mechanisms and Functional Interactions"* Kloster Seeon, Germany
2.	09/2002	4th Biannual Meeting of the Boehringer Ingelheim Fonds, Woodshole, Massachusetts
3.	05/2002	NIH conference *"The Lymphatic Continuum"*, NIH, Bethesda, Maryland
4.	02/2002	Cornell University, New York, New York
5.	01/2002	*"Tumor-Selective Therapy and Therapy Resistance"*, Essen, Germany
6.	10/2001	Annual Meeting of the Biomedical Engineering Society, Durham, North Carolina
7.	09/2001	University of Geneva, Geneva, Switzerland
8.	09/2001	XVIII International Congress of Lymphology, Genoa, Italy

Cells:

- human, GFP labeled MDA-MB-435/GFP breast cancer cell lines, low and highly lymphangiogenic (VEGF-C producing)

- human microvascular lymphatic endothelial cells and their blood endothelial counterparts

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7/01/2002-6/30/2005
Prognostic Significance of Lymphangiogenesis in Cancer
Role: PI

Pending

NIH/NCI, R01
VEGF-C-Mediated Lymphangiogenesis in Tumor Metastasis
Role: PI

SEARLE SCHOLARS PROGRAM
Molecular mechanisms of tumor cell interactions with lymphatic vessels
Role: PI

5. CONCLUSIONS

In conclusion, we have fully accomplished the goals of the study and have confirmed our hypothesis that induction of tumor lymphangiogenesis promotes the metastatic capability of breast cancer cells. We demonstrated the occurrence of intratumoral lymphangiogenesis within human breast cancers after orthotopic transplantation onto nude mice, and we have confirmed the relevance of these findings for human cancers, by showing that human breast cancers also exhibit an increase in lymphatic vessel density. Furthermore, we demonstrated that VEGF-C increased intratumoral lymphangiogenesis, resulting in significantly enhanced tumor metastasis to regional lymph nodes and to lungs. Importantly, the degree of tumor lymphangiogenesis was highly correlated with the extent of lymph node and lung metastases. These results establish the occurrence and biological significance of intratumoral lymphangiogenesis in breast cancer and identify VEGF-C as a molecular link between tumor lymphangiogenesis and metastasis.

6. REFERENCES:

1. M. Skobe et al., *Nat Med* 7, 192-198 (2001).
2. S. Podgrabinska et al., *Proc Natl Acad Sci U S A*, in press.

7. APPENDICES:

Copies of the pertinent articles
CV

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ACADEMIC APPOINTMENTS

06/2001-present	Assistant Professor	Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, NY
04/1999-05/2001	Research Assistant Instructor	Dept. of Dermatology, Harvard Medical School Molecular Biology/Dermatology, Massachussetts General Hospital Boston, MA

EDUCATION

Ph.D. 07/1996	University of Darmstadt&German Cancer Research Center, Heidelberg, Germany (cell biology)
B.S. 11/1991	University of Zagreb, Croatia (molecular biology)

POSTDOCTORAL TRAINING

01/1998-04/1999	Research Fellow	Dept. of Dermatology Harvard Medical School&Massachussetts General Hospital, Boston, MA
02/1997-01/1998	Research Fellow	Dept. of Pathology Harvard Medical School&Beth Israel Deaconess Medical Center, Boston, MA
07/1996-02/1997	Research Fellow	German Cancer Research Center, Heidelberg, Germany

HONORS

2001 Presidential Prize of the International Society of Lymphology

1998 Hermann-Rein Prize, awarded by the German Society for Microcirculation

1996 Bristol-Myers-Squibb Prize, awarded by the AACR

1996 Graduate Student Prize from the Metastasis Research Society

1993 Graduate Student Prize from the German Cancer Research Center

SOCIETIES AND OTHER ACTIVITIES

1998-present	European Society for Microcirculation
1999-present	AACR
2000-present	International Society of Lymphology

Editorial board of the journal *Lymphatic Research and Biology*

PUBLICATIONS

1. Podgrabinska S., Braun P., Velasco P., Kloos B., Pepper M.S., Jackson D.G. and **Skobe M.** Molecular characterization of lymphatic endothelial cells. *Proc. Natl. Acad. Sci. USA.* 2002; (in press)

2. Cassella M. and **Skobe M.** Activation of lymphatic vessels in cancer. *Ann NY Acad Sci* 2002; (in press)

3. Szuba A., **Skobe M.,** Karkkainen M.J., Shin W.S., Beyner D.P., Rockson N.B., Dakhil N., Spilman S., Goris M.L., Strauss H.W., Quartermous T., Alitalo K., Rockson S. Therapeutic lymphangiogenesis with human recombinant VEGF-C. *FASEB J* 2002; (in press)

4. Hawighorst T., **Skobe M.,** Streit M., Hong Y-K., Velasco P., Brown L.F., Riccardi L., Lange-Asschenfeldt B., Detmar M. Activation of the Tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth. *Am J Pathol.* 2002; 160(4):1381-92

5. Swartz M.A. & **Skobe M.** Lymphatic function, lymphangiogenesis and cancer metastasis. *Microscopy Res.* 2001; 55:92-99. Review.

6. **Skobe M.,** Hamberg L., Hawighorst T., Schirner M., Wolf G.L., Alitalo K., Detmar M. Concurrent induction of lymphangiogenesis, angiogenesis and recruitment of macrophages by VEGF-C in melanoma. *Am J Pathol.* 2001; 159(3):893-903

7. **Skobe M.,** Hawighorst T., Jackson D., Prevo R., Janes L., Velasco P., Riccardi L., Alitalo, K., Claffey K., Detmar M. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature Med.* 2001; 7:192-198

8. **Skobe M.** & Detmar M. Structure, function and molecular control of the skin lymphatic system. *J Invest Dermatol Proceed* 2000; 5(1):14-19. Review.

9. Detmar M., Velasco P., Richard L., Claffey K.P., Streit M., Riccardi L., **Skobe M.,** Brown L.F. Expression of vascular endothelial growth factor induces an invasive phenotype in human squamous cell carcinomas. *Am J Pathol.* 2000; 156(1):159-67

10. **Skobe M.,** Brown L.F., Tognazzi K., Ganju R.K., Dezube B.J., Alitalo K., Detmar M. Vascular endothelial growth factor-C (VEGF-C) and its receptors KDR and flt-4 are expressed in AIDS-associated Kaposi's sarcoma. *J Invest Dermatol.* 1999; 113(6):1047-53

11. Streit M., Velasco P., Brown L.F., **Skobe M.,** Richard L., Riccardi L., Lawler J., Detmar M. Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. *Am J Pathol.* 1999; 155(2):441-52

12. Bajou K., Noel A., Gerard R.D., Masson V., Brunner N., Holst-Hansen C., **Skobe M.,** Fusenig N.E., Carmeliet P., Collen D., Foidart J.M. Absence of the host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nature Med.* 1998; 4:923-928

13. **Skobe, M.** and Fusenig, N.E. Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. *Proc. Natl. Acad. Sci. USA.* 1998; 95:1050-1055

14. **Skobe, M.,** Vosseler, S., Rockwell, P., Goldstein, N., Fusenig, N.E. Halting angiogenesis supresses carcinoma cell invasion. *Nature Med.* 1997; 3:1222-1227

Book chapters

Fusenig N.E., **Skobe M.,** Vosseler S., Hansen M., Lederle W., Airola K., Tomakidi P., Stark H-J., Boukamp P., Breitkreutz D. Tissue models to study tumor stroma interactions. *in* "Proteases and their inhibitors in cancer metastasis" by J-M Foidart: (in press)

INVITED LECTURES

09/2002	*"Angiogenesis: Molecular Mechanisms and Functional Interactions"* Kloster Seeon, Germany
09/2002	4th Biannual Meeting of the Boehringer Ingelheim Fonds, Woodshole, Massachusetts
05/2002	NIH conference *"The Lymphatic Continuum"*, NIH, Bethesda, Maryland
02/2002	Cornell University, New York, New York
01/2002	*"Tumor-Selective Therapy and Therapy Resistance"*, Essen, Germany
10/2001	Annual Meeting of the Biomedical Engineering Society, Durham, North Carolina
09/2001	University of Geneva, Geneva, Switzerland

09/2001	XVIII International Congress of Lymphology, Genoa, Italy
05/2001	German Cancer Research Society Meeting, Cadenabbia/Como, Italy
04/2001	Harvard Medical School/Dana-Farber Cancer Institute, Boston, Massachusetts
03/2001	92nd Annual Meeting of the American Association for Cancer Research, New Orleans, Louisiana
02/2001	Harvard Medical School/Massachusetts General Hospital, Boston, Massachusetts
01/2001	Columbia University, New York, New York
12/2000	Stanford University, Stanford, California
10/2000	*"Angiogenesis Forum"*, German Cancer Research Center, Heidelberg, Germany
09/2000	3rd Biannual Meeting of the Boehringer Ingelheim Fonds, Woodshole, Massachusetts
05/2000	NIH Think-Tank *"Conquering Lymphatic Disease: Setting the Research Agenda"*, NIH Bethesda, Maryland
05/2000	61st Annual Meeting of the Society for Investigative Dermatology, Chicago, Illinois
10/1999	European School of Haematology: *"Angiogenesis and Tumors"*, Paris, France
08/1999	48th Annual Montagna Symposium on the Biology of the Skin: *"Endothelial cells and Angiogenesis: From Benchtop to Bedside"*, Snowmass, Colorado.
09/1996	6th International Congress of the Metastasis Research Society, Gent, Belgium.
04/1996	87th Annual Meeting of the American Association for Cancer Research, Washington DC

Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis

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Metastasis of breast cancer occurs primarily through the lymphatic system, and the extent of lymph node involvement is a key prognostic factor for the disease. Whereas the significance of angiogenesis for tumor progression has been well documented, the ability of tumor cells to induce the growth of lymphatic vessels (lymphangiogenesis) and the presence of intratumoral lymphatic vessels have been controversial. Using a novel marker for lymphatic endothelium, LYVE-1, we demonstrate here the occurrence of intratumoral lymphangiogenesis within human breast cancers after orthotopic transplantation onto nude mice. Vascular endothelial growth factor (VEGF)-C overexpression in breast cancer cells potently increased intratumoral lymphangiogenesis, resulting in significantly enhanced metastasis to regional lymph nodes and to lungs. The degree of tumor lymphangiogenesis was highly correlated with the extent of lymph node and lung metastases. These results establish the occurrence and biological significance of intratumoral lymphangiogenesis in breast cancer and identify VEGF-C as a molecular link between tumor lymphangiogenesis and metastasis.

The spread of tumor cells by lymphatic vessels to regional lymph nodes is an important indicator of tumor aggressiveness for most human malignancies. Lymphatic vessels containing clusters of tumor cells frequently occur at the periphery of malignant tumors; however, lymphatic vessels have been thought to be absent from tumors themselves[1–5]. Moreover, it is unclear whether tumors can stimulate lymphangiogenesis and whether activation of the lymphatic system may affect tumor progression and metastasis[5,6].

Though the significance of angiogenesis for tumor progression has been well documented, the molecular mechanisms regulating the growth and function of lymphatic vessels are largely unknown. Previously, vascular endothelial growth factor (VEGF)-C, a novel member of the VEGF family of angiogenic growth factors, was demonstrated to stimulate the growth of lymphatic vascular endothelium *in vivo*. Lymphangiogenesis is stimulated by VEGF-C in the avian chorioallantoic membrane assay[7], and transgenic mice overexpressing VEGF-C in the skin are characterized by specific hyperplasia of the lymphatic network[8]. In normal adult human tissues, the VEGF-C receptor VEGFR-3 (FLT-4) is predominantly expressed by lymphatic endothelia[9,10]. A second VEGF-C receptor, VEGFR-2 (KDR), is predominantly expressed by activated endothelia of blood vessels and is also used by VEGF (refs. 11,12). VEGF-D, structurally related to VEGF-C, also binds and activates VEGFR-2 and VEGFR-3 (ref. 13), indicating its function in the induction of lymphangiogenesis.

Expression of VEGF-C occurs in a variety of human tumors such as breast[14,15], colon[16,17], lung[15,18,19], thyroid[20–22], gastric[23] and squamous cell cancers[15], mesotheliomas[24], neuroblastomas[25], sarcomas[15] and melanomas[15]. Increased expression of its receptor VEGFR-3 has been detected in lymphatic endothelia adjacent to cancer cells and in lymph nodes containing carcinoma metastases[9,10]. Moreover, expression of VEGF-C mRNA has recently been shown to correlate with the rate of metastasis to lymph nodes in breast[14], colorectal[17], gastric[23], thyroid[21,22], lung[19] and prostate[26] cancers. To date, however, lymphangiogenesis has not been causally linked to tumor metastasis.

To directly assess the functional importance of lymphangiogenesis for cancer metastasis, we used genetically fluorescent MDA-MB-435/green fluorescent protein (GFP) human breast cancer cells transfected to overexpress VEGF-C in a nude mouse model of spontaneous breast cancer metastasis. Our results demonstrate, for the first time, the occurrence of intratumoral lymphangiogenesis in breast cancer and identify VEGF-C as a potent enhancer of tumor lymphangiogenesis, leading to increased metastatic spread of breast cancer cells to lymph nodes and lungs.

Overexpression of VEGF-C in human breast cancer cells

To investigate the role of lymphangiogenic factors in metastatic tumor spread, we transfected human MDA-MB-435 breast cancer cells, which reproducibly form metastases to regional lymph nodes and to lungs[27], so that they constitutively overexpress VEGF-C. First we established MDA-MB-435 cells that constitutively overexpress GFP (MDA-MB-435/GFP) to facilitate the detection of tumor micrometastases by fluorescence microscopy[28].

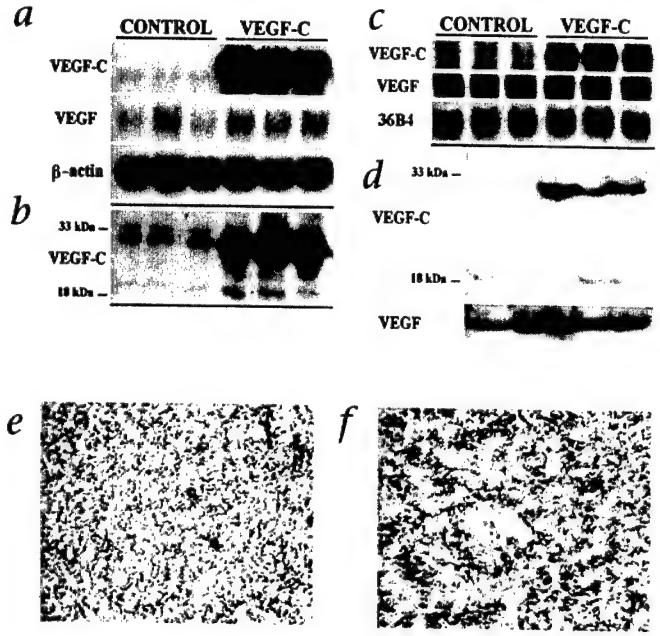


Fig. 1 Overexpression of VEGF-C in MDA-MB-435/GFP cells. ***a***, Northern-blot analysis of cultured MDA-MB-435/GFP clones confirmed strong overexpression of VEGF-C mRNA (2.4 kb) in VEGF-C–transfected clones, as compared with clones transfected with control vector. VEGF mRNA expression was unchanged. Hybridization with a β-actin cDNA probe was performed as a loading control. ***b***, Western-blot analysis of conditioned media confirmed abundant secretion of VEGF-C by MDA-MB-435/GFP–VEGF-C cells, while control transfectants secreted only low amounts of VEGF-C protein. The predominant VEGF-C form detected was the partially processed 31 kD form, whereas only small amounts of the fully processed 21 kD form were detectable. ***c***, Northern-blot analysis of total RNA obtained from tumors demonstrated continuing VEGF-C mRNA expression in VEGF-C–transfected MDA-MB-435/GFP tumors but little expression in control tumors. VEGF mRNA expression was comparable in both groups. Hybridization with ribosome-associated protein 36B4 cDNA served as loading control. ***d***, Western-blot analysis of tumor lysates confirmed efficient VEGF-C secretion in VEGF-C–transfected tumors. Predominantly, the 31-kD VEGF-C form was detected, with only trace amounts of the fully processed 21-kD form. Little VEGF-C was detected in control tumors, and VEGF was expressed at comparable levels in both control and VEGF-C–overexpressing tumors. ***e*** and ***f***, *In situ* hybridization with a human VEGF-C probe revealed that the majority of VEGF-C–transfected cells (*f*) maintained VEGF-C mRNA expression *in vivo* whereas little or no VEGF-C mRNA expression was detected in control tumors (*e*).

From this population we then selected a single clone for transfection with VEGF-C cDNA to reduce the variability in the metastatic potential between different cell clones. As determined by northern-blot analysis, vector-transfected control clones (MDA/GFP-control) expressed small amounts of VEGF-C mRNA, whereas three VEGF-C–transfected cell clones (MDA/GFP–VEGF-C) expressed high levels of VEGF-C mRNA *in vitro* (Fig. 1*a*). Western-blot analyses confirmed efficient secretion of VEGF-C in conditioned media obtained from MDA/GFP–VEGF-C cells (Fig. 1*b*). In contrast, only small amounts of VEGF-C protein were detectable in conditioned media obtained from control MDA-MB-435/GFP cells. Supernatants of MDA/GFP–VEGF-C cells contained mostly the 31 kD form of VEGF-C which activates VEGFR-3, and only small amounts of the 21 kD form, a high-affinity ligand for both VEGFR-2 and VEGFR-3 (ref. 29) (Fig. 1*b*). VEGF-C overexpression did not influence the expression of VEGF mRNA (Fig. 1*a*) *in vitro*. VEGF-D mRNA expression was undetectable whereas VEGF-D protein was expressed at comparable, low levels in control and VEGF-C–overexpressing clones (data not shown). VEGF-C overexpression did not modulate the proliferation of tumor cells *in vitro* (data not shown).

After orthotopic injection into the mammary fatpads of nude mice, parental, control and VEGF-C–transfected MDA-MB-435/GFP breast cancer cells had comparable growth rates *in vivo*, reaching an average tumor volume of approximately 1200 mm^3 within 12 weeks after injection. Northern-blot analysis of total RNA extracted from 12-week-old tumors confirmed strong continuing expression of VEGF-C mRNA in tumors derived from

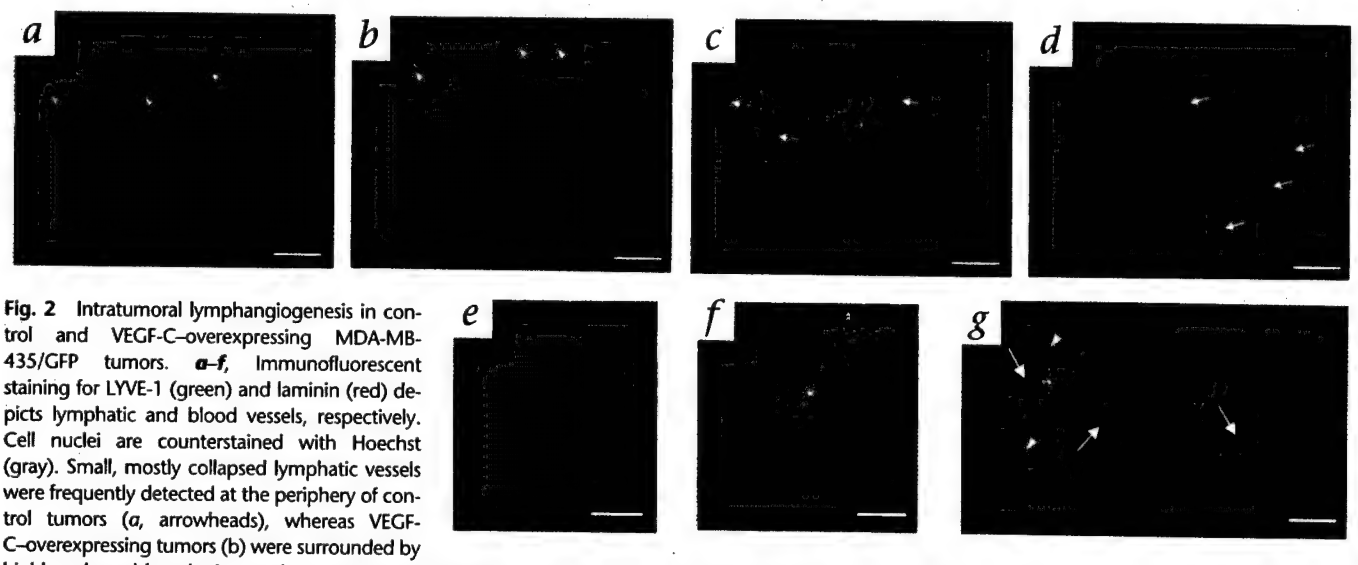


Fig. 2 Intratumoral lymphangiogenesis in control and VEGF-C–overexpressing MDA-MB-435/GFP tumors. ***a–f***, Immunofluorescent staining for LYVE-1 (green) and laminin (red) depicts lymphatic and blood vessels, respectively. Cell nuclei are counterstained with Hoechst (gray). Small, mostly collapsed lymphatic vessels were frequently detected at the periphery of control tumors (*a*, arrowheads), whereas VEGF-C–overexpressing tumors (b) were surrounded by highly enlarged lymphatic vessels. Intratumoral lymphatic vessels with open lumen were observed in control (*c*) and in VEGF-C–overexpressing tumors (*d*, arrows). Lymphatic vessel density was highly increased in central areas of VEGF-C–overexpressing tumors. Scale bars: 100 μm. Within VEGF-C–overexpressing tumors, lymphatic vessels with open lumina were regularly detected (*e*), frequently containing tumor cells (*f*). Scale bars, 50 μm. ***g***, Double immunofluorescent staining for LYVE-1 (green) and for the proliferation marker Ki67 (red) reveals proliferating lymphatic endothelial cells (arrows) within VEGF-C–overexpressing tumors. Proliferating tumor cells are labeled red (arrowheads). Scale bar, 25 μm.

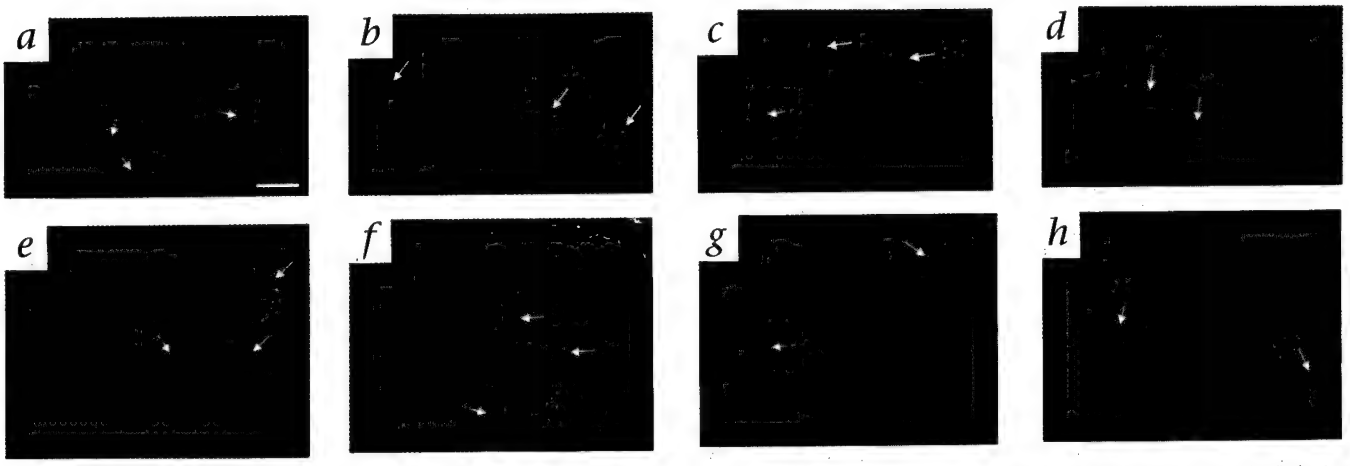
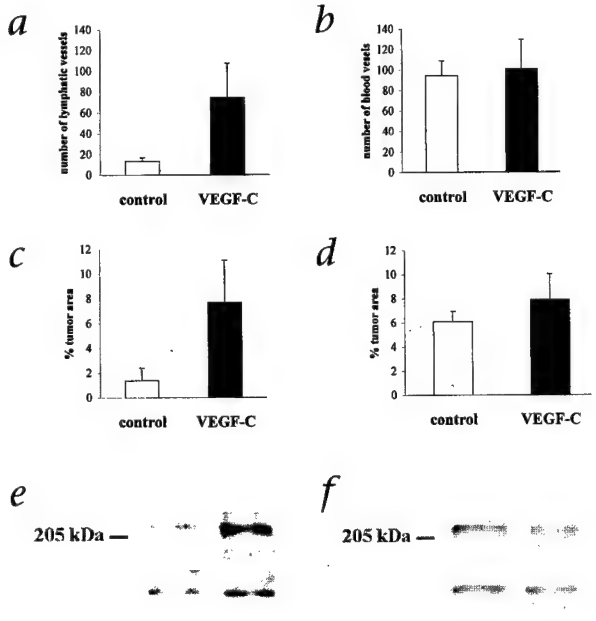


Fig. 3 Specific expression of LYVE-1 in lymphatic vessels of normal mouse skin (upper panel) and tumors (lower panel). ***a*** and ***e***, Double immunofluorescent staining with an anti-mouse LYVE-1 antibody (green) and an antibody against the endothelial junction molecule CD31 (red) demonstrated that only a fraction of all CD31-positive vessels expressed LYVE-1 (orange; arrows) in normal mouse skin (*a*) and in MDA-MB-435/GFP tumors (*e*). ***b*** and ***f***, Simultaneous staining for LYVE-1 (green) and VEGFR-3 (red) revealed identical expression patterns of both molecules in lymphatic vessels (orange; arrows) of normal skin (*b*) and tumors (*f*). ***c*** and ***g***, Staining for collagen XVIII (red) depicts basement membranes of the epidermal-dermal junction, hair follicles and blood vessels in normal skin (*c*) and of blood vessels in tumors (*g*). LYVE-1 expressing lymphatic vessels (green; arrows) did not express collagen XVIII. ***d*** and ***h***, Expression of laminin (red) was detected in basement membranes of the epidermal-dermal junction, hair follicles and blood vessels of normal skin (d) and of blood vessels in tumors (h). Laminin expression was largely absent from LYVE-1 positive lymphatic vessels (green; arrows). Scale bar: 100 μm.

VEGF-C–transfected MDA-MB-435/GFP cells, but only low-level expression in control tumors (Fig. 1*c*). These findings were confirmed by *in situ* hybridization studies that revealed increased VEGF-C mRNA expression in most VEGF-C–transfected tumor cells (Fig. 1*f*) but little or no expression in control tumors (Fig. 1*e*). Western-blot analyses revealed large amounts of secreted VEGF-C protein in lysates obtained from VEGF-C–overexpressing tumors but only little VEGF-C protein in control tumors (Fig. 1*d*). We primarily detected the secreted 31 kD form of VEGF-C (ref. 29), whereas we found only traces of the mature 21 kD VEGF-C form (Fig. 1*d*). We also detected the 21 kD form at comparable levels in lysates obtained from control tumors. These findings showed that only partial processing of VEGF-C occurred in MDA-MB-435/GFP tumors overexpressing VEGF-C *in vivo*. Overexpression of VEGF-C in MDA-MB-435/GFP tumors did not affect VEGF mRNA (Fig. 1*c*) or protein (Fig. 1*d*) expression *in vivo*, and we observed little or no VEGF-D mRNA or protein expression in control and VEGF-C–overexpressing tumors (data not shown).



VEGF-C increases intratumoral lymphangiogenesis

To visualize tumor-associated lymphatic vessels, we stained tissues with a newly derived antibody specific for the mouse LYVE-1 hyaluronan receptor. Like its human orthologue[30], mouse LYVE-1 is a highly specific marker of lymphatic vessels in a variety of different mouse tissues and in mouse lymphangiomas (Prevo *et al.*, manuscript submitted). Small lymphatic vessels with mostly compressed lumina were common in the skin surrounding control MDA-MB-435/GFP tumors (Fig. 2*a*), whereas lymphatic vessels were markedly enlarged in the peritumoral areas of VEGF-C–overexpressing tumors (Fig. 2*b*). Intratumoral lymphatic vessels with clearly detectable lumina were found in approximately 70% of control-transfected tumors, predominantly localized in the tumor periphery (Fig. 2*c*). In contrast, all VEGF-C–overexpressing tumors examined were infiltrated by

Fig. 4 VEGF-C overexpression induces intratumoral lymphangiogenesis. Quantitative computer-assisted image analysis revealed a dramatic increase in the density of lymphatic vessels within MDA-MB-435/GFP tumors overexpressing VEGF-C, as compared with control tumors (***a***; $P < 0.001$). In contrast, the density of blood vessels was not different between the two tumor types (***b***; $P = 0.45$). The relative tumor area occupied by lymphatic vessels was significantly increased in VEGF-C–overexpressing tumors (***c***; $P < 0.001$), whereas the relative area occupied by blood vessels was not significantly different between the two tumor types (***d***; $P = 0.5$). ***e***, Immunoprecipitation analysis of tumor lysates revealed increased amounts of VEGFR-3 (upper panel) and enhanced VEGFR-3 phosphorylation levels (lower panel) in VEGF-C expressing MDA-MB-435/GFP tumors (right lanes), as compared with control tumors (left lanes). ***f***, In contrast, comparable expression of VEGFR-2 (upper panel) and equal VEGFR-2 phosphorylation levels (lower panel) were detected in control (left lanes) and in VEGF-C–overexpressing tumors (right lanes).

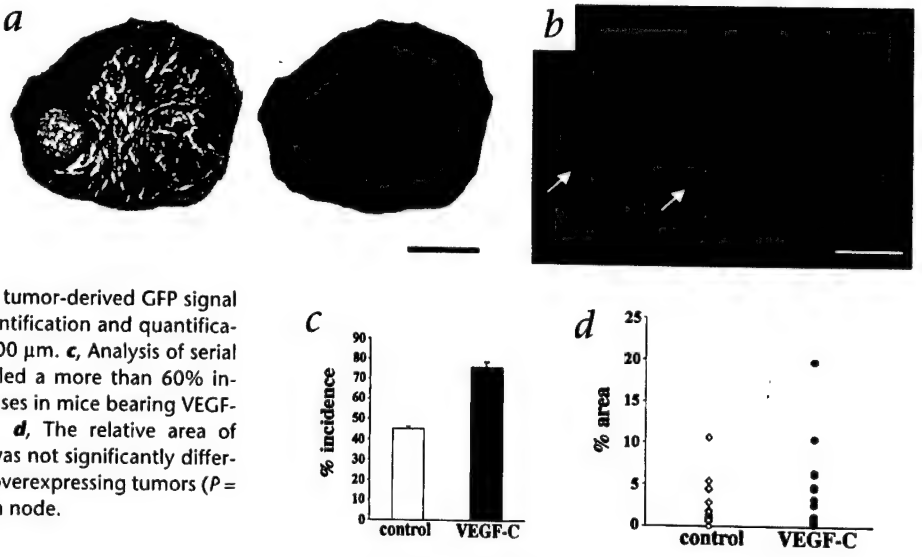


Fig. 5 VEGF-C increases the incidence of lymph node metastases. ***a***, Histology of a representative mouse lymph node containing breast cancer metastases (left). Fluorescence micrograph of a parallel section (right) reveals strong GFP expression by metastatic tumor cells. Note strict colocalization of metastatic foci, indicating that the majority of tumor cells in nonnecrotic areas expressed GFP. Scale bar = 1 mm. ***b***, Higher power fluorescence micrograph of a lymph node demonstrates that the tumor-derived GFP signal provides a highly sensitive method for the identification and quantification of micrometastases (arrows). Scale bar, 500 μm. ***c***, Analysis of serial sections through sentinel lymph nodes revealed a more than 60% increase in the incidence of lymph node metastases in mice bearing VEGF-C overproducing MDA-MB-435/GFP tumors. ***d***, The relative area of lymph nodes occupied by tumor metastases was not significantly different between mice bearing control or VEGF-C–overexpressing tumors ($P = 0.8$). Each data point represents a single lymph node.

numerous small lymphatic vessels (Fig. 2*d*). Whereas control tumors were invaded by lymphatic vessels to a maximum depth of 2 mm from the tumor–host interface, VEGF-C–overexpressing tumors were infiltrated throughout the central tumor areas, with lymphatic vessels extending up to 5 mm into the tumor. Within VEGF-C–overexpressing tumors, we regularly detected lymphatic vessels with open lumina that frequently contained tumor cells (Fig. 2*e* and *f*). Analysis of 100-μm thick tumor sections revealed that intratumoral lymphatics formed channels that were connected with lymphatic vessels at the tumor periphery (data not shown). We observed proliferating lymphatic endothelial cells within MDA-MB-435/GFP–VEGF-C tumors (Fig. 2*g*) and, less frequently, within control tumors.

We confirmed the specificity of LYVE-1 expression for lymphatic vessels in a series of differential immunofluorescent stainings of normal mouse skin and of MDA-MB-435/GFP–VEGF-C tumors, using the antibody against LYVE-1 in combination with several antibodies against endothelial antigens and basement membrane components. LYVE-1 was only expressed in a fraction of all vessels that expressed the endothelial junction molecule CD31 in both normal skin and in MDA-MB-435/GFP–VEGF-C tumors (Fig. 3*a* and *e*), and all LYVE-1$^+$ lymphatic vessels also expressed VEGFR-3 (Fig. 3*b* and *f*). In contrast, LYVE-1$^+$ vessels were mostly negative for collagen type XVIII (Fig. 3*c* and *g*) and for laminin (Fig. 3*d* and *h*), major components of the blood vascular basement membrane.

Computer-assisted image analysis of tumor sections stained for LYVE-1 revealed a 4.6-fold increase in the density of lymphatic vessels within VEGF-C–overexpressing tumors, as compared with control tumors ($P < 0.001$; Fig. 4*a*). The relative tumor area covered by lymphatic vessels within VEGF-C–overexpressing tumors increased correspondingly (Fig. 4*c*). In certain areas of the tumors, the lymphatic vessel density was as high as the density of tumor blood vessels (Fig. 4*b*). We did not observe any significant differences in the density of intratumoral blood vessels, or in the relative tumor area covered by blood vessels between VEGF-C–overexpressing and control tumors (Fig. 4*b* and *d*). These findings were confirmed by immunoprecipitation studies of tumor lysates that revealed similar expression and phosphorylation levels of VEGFR-2 in tumors of both types (Fig. 4*f*). In contrast, we found increased amounts of VEGFR-3 and enhanced VEGFR-3 phosphorylation levels in tumor lysates obtained from

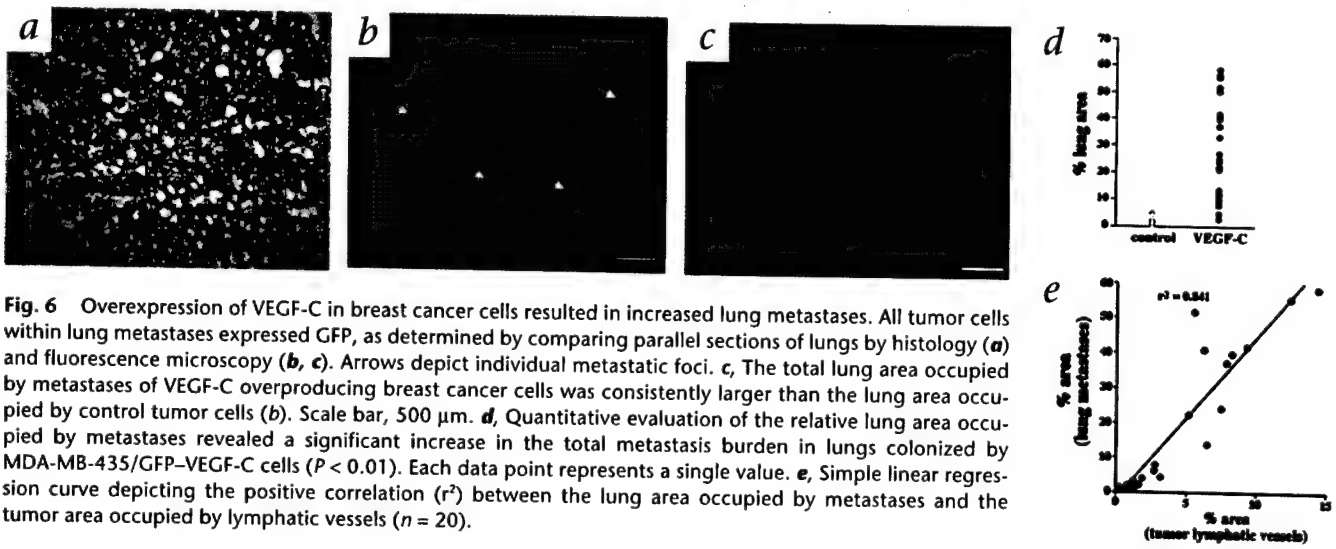


Fig. 6 Overexpression of VEGF-C in breast cancer cells resulted in increased lung metastases. All tumor cells within lung metastases expressed GFP, as determined by comparing parallel sections of lungs by histology (***a***) and fluorescence microscopy (***b***, ***c***). Arrows depict individual metastatic foci. ***c***, The total lung area occupied by metastases of VEGF-C overproducing breast cancer cells was consistently larger than the lung area occupied by control tumor cells (*b*). Scale bar, 500 μm. ***d***, Quantitative evaluation of the relative lung area occupied by metastases revealed a significant increase in the total metastasis burden in lungs colonized by MDA-MB-435/GFP–VEGF-C cells ($P < 0.01$). Each data point represents a single value. ***e***, Simple linear regression curve depicting the positive correlation (r^2) between the lung area occupied by metastases and the tumor area occupied by lymphatic vessels ($n = 20$).

VEGF-C–overexpressing tumors (Fig. 4*e*). Together, these results provide compelling evidence that lymphangiogenesis occurs within malignant tumors and that VEGF-C is a potent inducer of tumor lymphangiogenesis.

VEGF-C increases the incidence of lymph node metastases

Macroscopically, regional axillary lymph nodes were enlarged in all cases and the size of lymph nodes was not indicative of the presence of metastases. To obtain accurate quantitative analysis of metastases, we used breast cancer cells genetically labeled with GFP, a highly sensitive method for the direct visualization of micrometastases[28]. Histological examination of lymph nodes confirmed GFP expression in the majority of metastatic tumor cells within non-necrotic areas (Fig. 5*a* and *b*). Evaluation of serial sections of sentinel lymph nodes by fluorescence microscopy revealed that the incidence of GFP-expressing metastases was increased by more than 60% in mice bearing VEGF-C–overexpressing tumors, as compared with mice bearing control tumors (Fig. 5*c*). The relative area of lymph nodes containing metastases of control and VEGF-C–overexpressing MDA-MB-435/GFP cells was comparable, as determined by quantitative image analysis of affected lymph nodes (Fig. 5*d*). In summary, these results demonstrate that overexpression of VEGF-C in primary tumors resulted in enhanced rates of lymphatic metastasis.

VEGF-C increases lung metastases of breast cancer cells

We next investigated the effects of VEGF-C overexpression on the development of lung metastases. Fluorescence microscopy of lung sections at 12 weeks after tumor implantation showed that cells metastatic to lungs continued to express GFP at high levels (Fig. 6*b* and *c*). We found the presence of lung metastases in all the mice of both experimental groups; however, the total lung area containing metastases was larger in mice bearing VEGF-C–overexpressing tumors (Fig. 6*c*). Quantitative image analysis of lung metastases revealed that the relative lung area occupied by metastases was up to six times as large in mice bearing VEGF-C–overexpressing MDA-MB-435/GFP tumors, as compared with control tumors (≤ 60% and ≤ 10%, respectively; $P < 0.01$; Fig. 6*d*). Importantly, linear regression analysis of the relative lung area occupied by metastases compared with the tumor area occupied by lymphatic vessels demonstrated a strong positive correlation ($r^2 = 0.841$; Fig. 6*e*). These results clearly demonstrate that increased VEGF-C production in primary tumors resulted in an increase of lung metastases.

Discussion

Infiltration of lymphatic vessels by tumor cells has been found at the periphery of many experimental and human tumors, and the lymphatic system has been recognized as a conduit for tumor cell dissemination[31]. However, the presence and potential function of lymphatic vessels in tumors are unclear due to the lack of reliable molecular markers to distinguish the lymphatic vasculature from blood vessels[32]. Whereas some early studies reported intratumoral lymphatic vessels in certain types of cancer including breast cancer[33–35], this has been interpreted mainly as co-option of pre-existing lymphatic vessels by invading tumor cells, and it has been proposed that lymphatic vessels are absent from most tumors[1–6,36]. Moreover, evidence for an active molecular interaction of tumor cells with lymphatic vessels is still lacking, and whether tumors stimulate lymphangiogenesis is unclear[5,35].

Here, we reveal the occurrence of pronounced lymphangiogenesis within human metastatic breast cancers in nude mice, using a newly derived antibody to the mouse hyaluronan receptor LYVE-1 (ref. 30). We demonstrate that in normal skin and in tumors, LYVE-1 is expressed in vessels that exhibit traditional characteristics of lymphatics such as the lack of a continuous basement membrane[37], as shown by absence of expression of laminin and collagen type XVIII. Lymphatic vessels exhibited weak expression of the endothelial cell marker CD31 and all LYVE-1–expressing vessels were also positive for another lymphatic endothelial marker, VEGFR-3. Moreover, differential immunostaining with the mouse LYVE-1 antiserum and with an antibody against the blood vascular endothelial marker CD34 revealed that LYVE-1 was not detected on CD34-expressing vessels (data not shown and Prevo *et al.*, manuscript submitted). The expression of LYVE-1 on lymphatic vessels is in accordance with its presumed physiological function as a receptor involved in the transport of interstitial hyaluronan to the lymph nodes[30].

Our results demonstrate the presence of lymphangiogenesis within the peripheral areas of MDA-MB-435/GFP control tumors, suggesting the production of lymphangiogenic factors in this metastatic tumor. Overexpression of VEGF-C resulted in enlargement of peritumoral lymphatic vessels and in increased intratumoral lymphangiogenesis—identifying VEGF-C as a potent tumor lymphangiogenesis factor. We observed proliferation of lymphatic endothelial cells within VEGF-C–overexpressing tumors, and we detected anatomically well-defined lymphatic vessels throughout the tumors at a much higher density than in control tumors. The greater depth of lymphatic vessel invasion into VEGF-C–overexpressing tumors indicates a pronounced effect of VEGF-C on lymphatic endothelial cell migration *in vivo*. Whereas the overall lymphatic vessel density was fairly consistent within each tumor group, we found considerable regional heterogeneity within individual tumors, in particular within tumors overexpressing VEGF-C. This may reflect local variations of the tumor microenvironment such as differences in extracellular matrix composition or mechanical forces. Because of the unique structural and functional integration of lymphatic vessels with the interstitium that is critical for lymphatic function[37,38], a permissive extracellular microenvironment is also likely to be critical for lymphangiogenesis. Though we have preliminary evidence that lymphatic vessels are also frequently detectable within experimental VEGF-C–expressing malignant melanomas and within cutaneous squamous cell carcinomas induced by a chemical carcinogenesis regimen in mice (Skobe *et al.*, unpublished data), the presence of intratumoral lymphatics in spontaneously developing human tumors remains to be determined.

Overexpression of VEGF-C in MDA-MB-435/GFP tumors selectively induced tumor lymphangiogenesis but not tumor angiogenesis. The receptor specificity of VEGF-C and thus its biological function have been suggested to be regulated by differential proteolytic processing. The secreted 31 kD VEGF-C protein predominantly activates VEGFR-3 whereas the mature, fully processed 21 kD form additionally activates VEGFR-2 (ref. 29). Our results strongly support this concept. We found that the predominant VEGF-C form present in lysates of VEGF-C–overexpressing tumors was the 31 kD form whereas only traces of the 21 kD form were detectable, at levels similar to those in control tumors. Accordingly, we found marked induction of VEGFR-3 expression and phosphorylation in VEGF-

C–overexpressing tumors but no modulation of VEGFR-2 expression or phosphorylation. The selective activation of VEGFR-3 expressed by lymphatic vessels therefore provides a mechanistic explanation for the selective induction of tumor lymphangiogenesis by overexpression of VEGF-C in MDA-MB-435/GFP tumors.

Numerous studies have failed to identify functional lymphatics within tumors[6,39,40], leading to the concept that lymphangiogenesis may not play a major role in tumor progression to the metastatic phenotype[36]. However, the absence of detectable perfusion of tumor lymphatic vessels does not necessarily indicate absence of anatomically distinguishable lymphatic vessels from tumors. Moreover, though the functional state of tumor-associated lymphatic vessels with respect to the efficient transport of fluids and macromolecules is of great importance for overall tumor physiology and drug delivery[41], it may not be critical for tumor dissemination. Indeed, the formation of an intratumoral lymphatic network, whether functional in fluid transport or not, might promote metastatic tumor spread by creating increased opportunities for metastatic tumor cells to leave the primary tumor site.

This concept is strongly supported by our findings that the increased density of lymphatic vessels within VEGF-C–overexpressing breast cancers was associated with a significantly increased—more than 60% higher—incidence of metastases in regional lymph nodes. Therefore, our findings provide a mechanistic explanation for the recently reported correlation of VEGF-C expression in the primary tumors with high incidence of lymph node metastases in breast, colorectal, gastric, thyroid, lung, and prostate cancers[14,17,19,21–23,26]. Moreover, a recent study found that VEGF-C expression was only detectable in node-positive breast cancers, whereas expression of VEGF was detected in both node-positive and node-negative tumors[14]. VEGF-C expression and lymphangiogenesis in these tumors have yet to be clearly correlated. However, though VEGF-C may promote the incidence of lymphatic metastases simply by increasing the number of lymphatic vessels in the vicinity of tumor cells, it is possible that activation of lymphatics by VEGF-C or related factors promotes molecular interactions of tumor cells with lymphatic endothelial cells, thereby facilitating tumor cell entry into the lymphatics.

VEGF-C overexpression in primary tumors resulted in a significant increase of lung metastases. This was most likely not due to accelerated growth rates of metastatic MDA-MB-435/GFP–VEGF-C cells in the lung, since VEGF-C overexpression did not confer a growth advantage to tumor cells at either the primary tumor site or in the lymph nodes. Moreover, we did not see an increase in tumor angiogenesis in VEGF-C expressing tumors, so it is unlikely that an increased rate of dissemination through blood vessels accounted for the increase in lung metastases. Importantly, the extent of intratumoral lymphatic vessel density was highly correlated with the extent of lung metastases, indicating an important role of the lymphatic system in distant metastatic tumor spread. From the lymph node, tumor dissemination can occur via efferent lymphatics or through lymphatic-venous communications within the nodes, and subsequently via the blood stream[31]. Although the exact pathway of breast cancer metastases to the lungs is unknown, our results indicate an increased incidence of lung colonization by tumor cells leaving the primary tumor site through the lymphatics. Because the lymphatic system is a low-pressure and low-flow system that is physiologically optimally adapted for the transport of cells[35], the preferential metastasis via lymphatics, due to expression of lymphangiogenic factors, might promote survival of disseminating tumor cells and thus increase their metastatic efficiency.

Our findings identify, for the first time, an active, growth factor-mediated interaction between tumor cells and tumor-associated lymphatic vessels with important implications for the formation of regional and distant metastases. An improved understanding of the molecular mechanisms that control metastatic tumor spread may enable early recognition of the metastatic potential of primary cancers and the development of new therapeutic strategies for limiting cancer spread.

Methods

Cell transfections. An *EcoRI-XbaI* EGFP-N2 fragment of the pEGFP-N2 vector (Clontech, Palo Alto, California) was subcloned into the pcDNA3.1/Neo vector (Invitrogen, San Diego, California). MDA-MB-435 (ref. 27) cells (American Type Culture Collection, Rockville, Maryland) grown in DMEM with 10% FBS were transfected with this expression construct (pcDNA3.1/EGFP) using the Superfect reagent (Qiagen, Chatsworth, California) and selected with Geneticin. MDA-MB-435/GFP clone 6 that exhibited tumor take of 100% and reliably produced lymph node and lung metastases was transfected with the human VEGF-C cDNA (ref. 11) cloned into a pcDNA3.1/Zeo expression vector or with the vector alone. Stably-transfected cell lines were maintained in media containing 600 μg/ml Zeocin and 400 μg/ml Geneticin.

Northern- and western-blot analyses. Northern-blot analyses were performed as described[42]. For western analyses, conditioned media from subconfluent cells grown for 60 hours serum-free were concentrated 100-fold, using Centricon-10 columns (Amicon, Beverly, Massachusetts). Tumors were snap frozen in liquid nitrogen and homogenized in lysis buffer (2% SDS, 50 mM Tris pH 7.4, 0.02M phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin and 50 μg/ml aprotinin). 15 μg of protein per sample was analyzed by denaturing SDS/PAGE and immunoblotted with antibodies against human VEGF-C (ref. 29) or VEGF (Neomarkers, Freemont, California). For receptor phosphorylation assays, tumors were homogenized in lysis buffer containing 20 mM Tris pH 7.6, 150 mM NaCl, 50 mM NaF, 1 mM Na-orthovanadate, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors as above. Protein samples (2 mg) were immunoprecipitated using antibodies against mouse VEGFR-2 or VEGFR-3 (Santa Cruz Biotechnology, Santa Cruz, California) and western analysis performed as above using antibodies against phosphotyrosine (PY-20, ICN Biomedicals, Aurora, Ohio), VEGFR-2 or VEGFR-3.

Metastasis assay. Cells were injected bilaterally into the second mammary fat pads of athymic, female, 8-wk-old NCR nu/nu mice (2×10^6 cells/100 μl serum-free culture medium). Mice were killed after 12 wk. 3 cell clones were analyzed for each construct (control, VEGF-C), 10 mice per clone. To reduce intrinsic background fluorescence of the lungs, mice were perfused post-sacrifice through the heart with PBS. Lungs were perfused through the trachea with 10% buffered formalin, fixed for 1 h in 10% formalin, washed in PBS, embedded in OCT and frozen in liquid nitrogen. Two sentinel lymph nodes were removed from each mouse (1 on each side) and processed as above. Tumors were embedded in OCT and frozen in liquid nitrogen.

Immunostainings and *in situ* hybridization. Cryosections were stained as previously described[43], using antibodies to mouse LYVE-1, collagen type XVIII (from R. Timpl), CD31 (Pharmingen), VEGFR-3 (R&D Systems), Ki67 (Novocastra), laminin (Chemicon), and corresponding secondary antibodies labeled with AlexaFluor488 (Molecular Probes, Eugene, Oregon) or Texas Red (Jackson Labs, West Grove, Pennsylvania). Cell nuclei were counterstained with 20 μg/ml Hoechst bisbenzimide (Sigma-Aldrich). Specimens were examined by using a Nikon E-600 microscope and images captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, Michigan). Ki67 immunostains were examined using a Leica DM IRBE microscope and a Leica TCS 4D confocal system. Non-radioactive *in*

situ hybridization was performed as described[44].

Morphometric analysis of intratumoral vasculature. Tumor sections were double-stained with antibodies to LYVE-1 and CD31, to visualize lymphatic (LYVE-1$^+$) and blood (CD31$^+$/LYVE-1$^-$) vessels. 2 clones were evaluated per tumor type, 5 tumors per clone. 5 areas of each tumor were evaluated at ×6 magnification. Lymphatic and blood vessels were quantified in tumor areas with the highest density of lymphatic vessels ('hot spots'), using the IPLab software (Scanalytics, Billerica, Massachusetts). The depth of lymphatic vessel infiltration into the tumors was determined by measuring the shortest distance from the tumor-host interface to the innermost lymphatic vessel. The unpaired Student's *t*-test was used for statistical analyses.

Quantitative evaluation of metastases. Lymph nodes and lungs from 10 mice for each of three cell clones per tumor type (control, VEGF-C) were evaluated by fluorescence microscopy. Five, 100 μm thick serial sections were examined per lymph node at ×2.4 magnification. The incidence of metastases was calculated for each cell clone as the number of positive lymph nodes per total number of lymph nodes examined, and the results expressed as the mean of 3 different cell clones per tumor type ± s.d. The unpaired Student's *t*-test was used for statistical analyses. For each cell clone, the size of the tissue area containing metastases was determined in five lymph nodes bearing metastases, using the IPLab software.

The size of lung metastases was determined in 10, 100-μm thick step sections (a total lung thickness of 2 mm examined) at ×2.4 magnification in the areas with the highest density of metastases. To calculate the correlation coefficient between the size of lung metastases and the size of the area occupied by lymphatic vessels in tumors, a simple linear regression analysis was performed on 20 samples (10 for each tumor group). Results are representative of two experiments.

Acknowledgments

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1. Lee, F.C. & Tilghmann, R.C. Lymph vessels in rabbit carcinoma, with a note on the normal lymph vessel structure of the testis. *Arch. Surg.* **26**, 602–616 (1933).
2. Gilchrist, R.K. Surgical management of advanced cancer of the breast. *Arch. Surg.* **61**, 913–929 (1950).
3. Zeidman, I., Copeland, B.E. & Warren, S. Experimental studies on the spread of cancer in the lymphatic system. II. Absence of a lymphatic supply in carcinomas. *Cancer* **8**, 123–127 (1955).
4. Tanigawa, N. *et al.* Experimental study on lymphatic vascular changes in the development of cancer. *Lymphology* **14**, 149–154 (1981).
5. Folkman, J. Angiogenesis and tumor growth. *N. Engl. J. Med.* **334**, 921 (1996).
6. Leu, A.J., Berk, D.A., Lymboussaki, A., Alitalo, K. & Jain, R.K. Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res.* **60**, 4324–4327 (2000).
7. Oh, S.J. *et al.* VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev. Biol.* **188**, 96–109 (1997).
8. Jeltsch, M. *et al.* Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* **276**, 1423–1425 (1997).
9. Kaipainen, A. *et al.* Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc. Natl. Acad. Sci. USA* **92**, 3566–3570 (1995).
10. Jussila, L. *et al.* Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res.* **58**, 1599–1604 (1998).
11. Joukov, V. *et al.* A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J.* **15**, 1751 (1996).
12. Neufeld, G., Cohen, T., Gengrinovitch, S. & Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* **13**, 9–22 (1999).
13. Achen, M.G. *et al.* Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc. Natl. Acad. Sci. USA* **95**, 548–553 (1998).
14. Kurebayashi, J. *et al.* Expression of vascular endothelial growth factor (VEGF) family members in breast cancer. *Jpn. J. Cancer Res.* **90**, 977–981 (1999).
15. Salven, P. *et al.* Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. *Am. J. Pathol.* **153**, 103–108 (1998).
16. Andre, T. *et al.* VEGF, VEGF-B, VEGF-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. *Int. J. Cancer* **86**, 174–181 (2000).
17. Akagi, K. *et al.* Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br. J. Cancer* **83**, 887–891 (2000).
18. Niki, T. *et al.* Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. *Clin. Cancer Res.* **6**, 2431–2439 (2000).
19. Ohta, Y., Nozawa, H., Tanaka, Y., Oda, M. & Watanabe, Y. Increased vascular endothelial growth factor and vascular endothelial growth factor-c and decreased nm23 expression associated with microdissemination in the lymph nodes in stage I non-small cell lung cancer. *J. Thorac. Cardiovasc. Surg.* **119**, 804–813 (2000).
20. Shushanov, S. *et al.* VEGF-C and VEGFR3 expression in human thyroid pathologies. *Int. J. Cancer* **86**, 47–52 (2000).
21. Bunone, G. *et al.* Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. *Am. J. Pathol* **155**, 1967–1976 (1999).
22. Fellmer, P.T. *et al.* Vascular endothelial growth factor-C gene expression in papillary and follicular thyroid carcinomas. *Surgery* **126**, 1056–1061 (1999).
23. Yonemura, Y. *et al.* Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin. Cancer Res.* **5**, 1823–1829 (1999).
24. Ohta, Y. *et al.* VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. *Br. J. Cancer* **81**, 54–61 (1999).
25. Eggert, A. *et al.* High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. *Clin. Cancer Res.* **6**, 1900–1908 (2000).
26. Tsurusaki, T. *et al.* Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. *Br. J. Cancer* **80**, 309–313 (1999).
27. Price, J.E., Polyzos, A., Zhang, R.D. & Daniels, L.M. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res.* **50**, 717–721 (1990).
28. Hoffman, R.M. Orthotopic transplant mouse models with green fluorescent protein-expressing cancer cells to visualize metastasis and angiogenesis. *Cancer Metastasis Rev.* **17**, 271–277 (1998).
29. Joukov, V. *et al.* Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J.* **16**, 3898–3911 (1997).
30. Banerji, S. *et al.* LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J. Cell Biol.* **144**, 789–801 (1999).
31. Fisher, B. & Fisher, E.R. Role of the lymphatic system in dissemination of tumor. in *Lymph and the lymphatic system* (ed. Mayerson, H.S.) 324 (Charles C. Thomas, Springfield, Illinois, 1968).
32. Skobe, M. & Detmar, M. Structure, function and molecular control of the skin lymphatic system. *J. Invest. Dermatol. Symp. Proceed.* **5**, 14–19 (2000).
33. Evans, H.M. On the occurence of newly-formed lymphatic vessels in malignant growths. *John Hopkins Med. J.* **19**, 232 (1908).
34. Reichert, F.L. The regeneration of the lymphatics. *Arch. Surg.* **13**, 871–881 (1926).
35. Witte, M.H., Way, D.L., Witte, C.L. & Bernas, M. Lymphangiogenesis: mechanisms, significance and clinical implications. *EXS* **79**, 65–112 (1997).
36. Carmeliet, P. & Jain, R.K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249–257 (2000).
37. Leak, L.V. Electron microscopic observations on lymphatic capillaries and the structural components of the connective tissue-lymph interface. *Microvasc. Res.* **2**, 361–391 (1970).
38. Pullinger, B.D. & Florey, H.W. Some observations on the structure and functions of lymphatics: their behavior in local edema. *Br. J. Exp. Pathol.* **16**, 49 (1935).
39. Jain, R.K. Transport of molecules in the tumor interstitium: a review. *Cancer Res.* **47**, 3039–3051 (1987).
40. Baxter, L.T. & Jain, R.K. Transport of fluid and macromolecules in tumors. II. Role of heterogeneous perfusion and lymphatics. *Microvasc. Res.* **40**, 246–263 (1990).
41. Jain, R.K. Vascular and interstitial barriers to delivery of therapeutic agents in tumors. *Cancer Metastasis Rev.* **9**, 253–266 (1990).
42. Detmar, M. *et al.* Expression of vascular endothelial growth factor induces an invasive phenotype in human squamous cell carcinomas. *Am. J. Pathol.* **156**, 159–167 (2000).
43. Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S. & Fusenig, N.E. Halting angiogenesis suppresses carcinoma cell invasion. *Nature Med.* **3**, 1222–1227 (1997).
44. St Croix, B. *et al.* Genes expressed in human tumor endothelium. *Science* **289**, 1197–1202 (2000).

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Lymphatic Function, Lymphangiogenesis, and Cancer Metastasis

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ABSTRACT The lymphatic system serves as the primary route for the metastasis of many cancers and the extent of lymph node involvement is the most important indicator of tumor aggressiveness. Despite the apparent importance of the lymphatic vessels for tumor dissemination, it has remained unclear whether activation of lymphatic endothelial cells may affect tumor progression and metastasis and the molecular mechanisms of lymphangiogenesis are just beginning to be elucidated. This overview describes the unique structural and functional characteristics of the lymphatic vessels that render them particularly suitable for invasion by tumor cells and for their efficient transport to lymph nodes. Recent evidence indicates occurrence of tumor lymphangiogenesis and its correlation with metastasis. Molecular regulation of tumor lymphangiogenesis, its significance for tumor metastasis, and implications for cancer therapy are discussed. *Microsc. Res. Tech. 55:92–99, 2001.*

INTRODUCTION

The lymphatic and blood vascular system, although structurally two distinct systems, are functionally interconnected and act in concert to maintain tissue homeostasis. The lymphatic system in many ways complements functions of the blood vascular system by regulating tissue fluid balance, facilitating interstitial protein transport, and serving immunological functions. Whereas mechanisms of angiogenesis involving blood vessels have been studied extensively over the past years, mostly due to the importance of angiogenesis in tumor growth and metastasis, little effort has been directed toward understanding regulatory mechanisms of lymphatic vessel growth and function in physiological and pathological conditions. Meanwhile, the lymphatic system is the primary route for the dissemination of many cancers and the extent of lymph node involvement is a key prognostic factor for the outcome of the disease; despite this, the major issues regarding the involvement of lymphatic vessels in tumor progression have remained unresolved.

The lymphatic vessels comprise a one-way transport system for fluid and proteins by collecting them from the interstitial space and returning to the blood circulation. As blood travels into the capillaries, plasma fluid and proteins extravasate into the interstitial space according to hydrostatic and osmotic pressure gradients. Most of this fluid gets reabsorbed into postcapillary venules, but osmotic forces resulting from the extravasated proteins cause a small net fluid flux into the tissue. The lymphatic capillaries drain this net exudate and therefore facilitate convective protein transport through the interstitium (Aukland and Reed, 1993; Schmid-Schönbein, 1990b). If the lymphatics become blocked or dysfunctional, interstitial protein accumulates, leading to continual increase of osmotic pressure and thus fluid accumulation (edema) ensues. The net fluid efflux from the blood, and therefore the net flow rate of lymph, is about two to three orders of magnitude less than the flow rate of the blood. Because of the high permeability of the lymphatic capillaries, the composition of lymph is nearly equivalent to that of interstitial fluid, which in turn is similar to, but less concentrated than, that of blood plasma. Intestinal lymph, in addition, contains a high amount of lipids resorbed directly from the intestine. The simplified relation between blood, interstitium, and lymph is depicted in Figure 1.

Lymphatic vessels and the lymph nodes are also important components of the immune system. Lymphatic vessels direct antigen-presenting cells to the lymph nodes and are thus essential for the development of cellular immunity. In the skin, for example, lymphatic vessels are an exit path for Langerhans cells. Impairment of lymphatic functioning, e.g., inadequate transport of fluid, macromolecules, or cells from the interstitium, leads to a number of diseases that are characterized by edema, impaired immunity, and fibrosis (Mortimer et al., 1990).

ORGANIZATION AND STRUCTURAL CHARACTERISTICS OF THE LYMPHATIC SYSTEM

There are five main categories of conduits in the lymphatic system: the lymphatic capillaries, collecting vessels, lymph nodes, lymphatic trunks, and ducts, whose sizes range from 10 μm to 2 mm in diameter. Lymph forms when interstitial fluid moves into the

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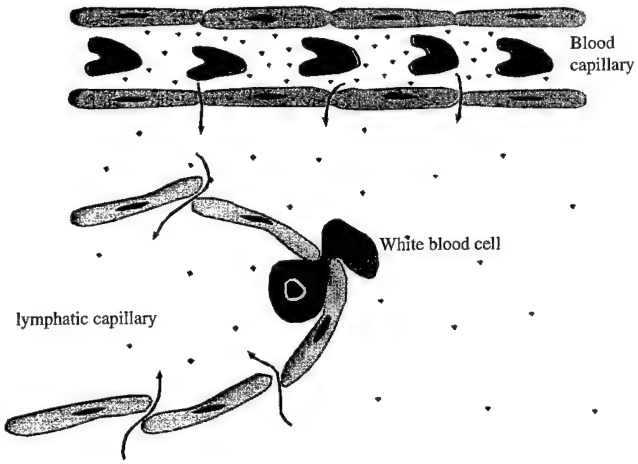

Fig. 1. Relationship between the blood and lymphatic capillaries.

lymphatic capillaries. From the capillaries it drains into the collecting vessels, which pass through at least one but usually through several clusters of lymph nodes. Collecting vessels drain into larger trunks, which lead into the lymphatic ducts. Finally, the lymphatic ducts return the lymph back into the bloodstream, completing the circuit of fluid transport.

Lymphatic capillaries (also called initial or terminal lymphatics) are blind-ended structures that are optimally suited for fluid and particle uptake. Similar to blood capillaries, lymphatics are comprised of a single nonfenestrated endothelial cell layer, but the structure of lymphatic capillaries is different from that of blood capillaries in several important aspects (Casley-Smith and Florey, 1961; Daroczy, 1988; Leak, 1970). They generally possess a more irregular and wider lumen (10–60 μm in diameter) than blood capillaries and their endothelium is typically characterized by an extremely attenuated cytoplasm, except in the perinuclear region. In contrast to blood vessels, lymphatic capillaries have absent or poorly developed basal lamina and they are not encircled by pericytes. Tight junctions and adherens junctions, the major types of intercellular junctions in blood vessels, are not as frequently seen in lymphatics. While these junctions in blood vessels are typically implicated in maintaining firm cell–cell adhesion to connect adjacent endothelial cells over entire cell boundaries, in lymphatics they represent focal points of adhesion instead. Finally, one of the most striking features of lymphatic capillaries is their intimate association with the adjacent interstitial areas. Lymphatic endothelial cells are closely connected to the surrounding tissue by fine strands of elastic fibers (Gerli et al., 1991; Pullinger and Florey, 1935). These anchoring filaments are attached to the abluminal surface of the cells and extend deeply into the connective tissue, thereby firmly attaching lymphatic endothelium to extracellular matrix fibers. Lymphatic endothelial cells are also characterized by numerous invaginations and cytoplasmic vesicles on both luminal and abluminal surfaces that are involved in transendothelial transport of molecules into the lumen (Cornford and Oldendorf, 1993; Leak, 1976; Marchetti et al., 1991).

From the lymphatic capillaries, lymph drains into the collecting lymphatics. Unlike the initial lymphatics, the collecting vessels are generally not tethered to the extracellular matrix, but instead contain smooth muscle and thus may support a circumferential hoop stress (Aukland and Reed, 1993; Schmid-Schönbein, 1990b). They also contain one-way valves that aid in lymph propulsion and prevent retrograde flow. Segments of collecting lymphatics between valves are termed lymphangions; each lymphangion serves as a contractile compartment that propels lymph into the next compartment. All collecting lymphatics pass through the lymph nodes and can be further classified as prenodal (afferent) or postnodal (efferent), to specify whether they carry lymph to or from the lymph nodes. Lymph nodes are compartmentalized into narrow fluid crevices where blood and lymphatic compartments oppose each other for fluid exchange and cell transport (Schmid-Schönbein, 1990b). From the final set of lymph nodes, lymphatic trunks drain lymph into the lymphatic ducts. The thoracic duct is the final branch of the lymphatic system that enters the lower region of the chest by passing through the aortic opening of the diaphragm; it drains into blood via the junction of the left jugular and subclavian veins.

Although lymphatic vessels are often found in proximity to blood vessels in tissues, the density of lymphatic plexus does not always match the abundance of blood supply. For example, there are no lymphatic vessels in the central nervous system and lymphatic vessels do not penetrate as far as blood vessels in several other well-vascularized tissues. In lobular organs such as the liver and mammary glands, lymphatic capillaries do not penetrate the lobules but instead surround their periphery. In skeletal muscle they are confined only to the fascial planes. Other tissues, such as the cornea of the eye and cartilage, are devoid of both blood and lymphatic vessels. Lymphatic-rich tissues include the skin, lung, and gastrointestinal tract. (Yoffey and Courtice, 1970).

MECHANISMS OF LYMPH FORMATION

Mammalian lymphatic capillaries contain no smooth muscle and are generally observed in a partially or fully collapsed state (Aukland and Reed, 1993; Schmid-Schönbein, 1990a). To function, they are critically dependent on their connections to the extracellular matrix by anchoring filaments. These fibers, 6–10 nm in diameter, are composed of elastin similar to that found in the extracellular matrix (Gerli et al., 1990) and tether the endothelium to adjacent collagen fibers (Leak and Burke, 1966). Thus, they are highly sensitive to interstitial stresses. An increase in the interstitial fluid volume (i.e., strain or swelling of the extracellular matrix) causes the anchoring filaments to exert radial tension on the lymphatic capillary to 'pull it open' or increase its luminal volume (Aukland and Nicolaysen, 1981; Aukland and Reed, 1993; Bert et al., 1988; Hogan and Unthank, 1986) (Fig. 2). This creates a "tissue pump," or a small oscillating pressure gradient, which facilitates lymph formation (Ikomi and Schmid-Schönbein, 1996; Schmid-Schönbein, 1990a). The concept of anchoring filaments helps to explain why venules are often compressed in inflammation and other conditions associated with tissue edema, while

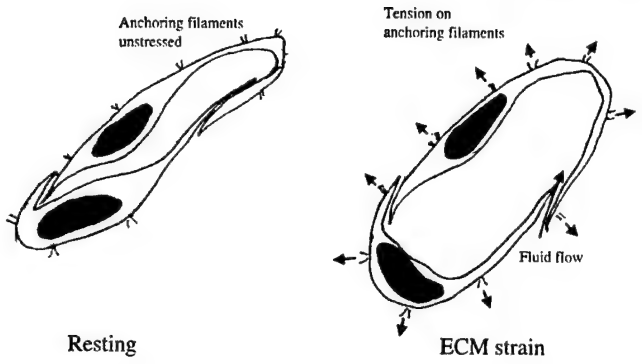

Fig. 2. The "tissue pump" that enables lymph formation: stress within the interstitium creates radial tension on the anchoring filaments, locally increasing the luminal volume of the lymphatic capillary. This creates a slight and temporary pressure difference, driving interstitial fluid into the lymphatic vessel through the passages formed by opening of overlapping endothelial cell junctions.

lymphatic capillaries are typically dilated (Pullinger and Florey, 1935). However, the dependence of lymph formation rates on local tissue pressure or volume diminishes at high interstitial fluid volumes (Guyton, 1965; Taylor et al., 1973) and is most likely limited by systemic forces that drive lymph propulsion. Overall, the functional state of lymphatic vessels cannot be necessarily determined by the vessel morphology, since an open lumen can indicate vessels both with dysfunction as well as normal function but increased load.

Lymph drainage is also accommodated by the opening of the intercellular junctions. Overlapping intercellular junctions formed by extensive superimposing of adjacent endothelial cells are a property unique to lymphatic vessels. By being loosely apposed to each other over long distances, lymphatic endothelial cells cast intercellular clefts. As the interstitium swells, anchoring filaments not only increase the vessel lumen, but also pull open the intercellular junctions to permit easy passage of fluids and particles into the vessel (Fig. 2). As fluid enters the lumen and decreases the pressure difference across the vessel wall the junctions begin to close, thereby preventing retrograde flow back into the interstitium (Ikomi and Schmid-Schönbein, 1996; Schmid-Schönbein, 1990b).

The extracellular matrix therefore plays an integral role in lymphatic function, as fluid equilibrium is controlled by the cooperation of both lymphatic function and the extracellular matrix. The elasticity and hydration of a tissue is determined by the composition and organization of the extracellular matrix; e.g., collagen provides structural framework and proteoglycans largely determine water content and resistance to fluid transport. Extensive and chronic degradation of the extracellular matrix eventually renders lymphatic vessels nonresponsive to the changes in the interstitium and therefore causes dysfunction (Negrini et al., 1996). In light of its importance in lymphatic function (i.e., the interstitial–lymphatic interface most clearly differentiates lymphatic from blood vascular capillaries), the composition and architecture of the ECM are likely to play a critical role in lymphangiogenesis and should be taken into consideration when studying the biology and pathology of the lymphatic system.

LYMPH TRANSPORT THROUGH THE LYMPHATICS

Transport of lymph through the lymphatic system (lymph propulsion) is coupled to lymph formation and both components contribute to the net flow rate in the lymphatics. The term "formation" describes fluid transport from the interstitium into the initial lymphatics and "propulsion" refers to the systemic forces that drive lymph from the initial capillaries to the larger vessels and eventually back to the blood. If there is blockage in the systemic route (e.g., removal of a lymph node), interstitial fluid may enter the initial lymphatics but will eventually "back up" as fluid is not drained from them, causing edema. Likewise, if the interstitial–lymphatic interface is destroyed and lymphatic capillaries cannot function, no lymph will be drained from that local region despite the baseline systemic drainage forces.

The driving forces for lymph formation are local: namely, interstitial fluid pressure and strain of the extracellular matrix and can be affected by skeletal motion and massage as well as the slight strains associated with pressure oscillations caused by arterial pressure pulsations and vasomotion of neighboring arterioles. The forces that drive lymph propulsion through the lymphatics, on the other hand, include systemic forces such as respiration (Negrini et al., 1994; Schad et al., 1978; Schmid-Schönbein, 1990b; Swartz et al., 1996), blood pressure (Parsons and McMaster, 1938), exercise (Olszewski et al., 1977), and massage (Ikomi and Schmid-Schönbein, 1996; McGeown et al., 1988; Mortimer et al., 1990), and are largely independent of the lymph formation rate.

The measurements of lymph flow velocity that have been reported in the literature are limited to superficial vessels in organs that can be visualized by in vivo microscopy. In the human skin lymph flow velocity averages 10 μm/s (Fischer et al., 1994); in the tail skin of anesthetized mice 3 μm/s (Berk et al., 1996; Swartz et al., 1996). However, lymph flow seems to fluctuate and oscillate, with a broad range of velocities of up to ±20 times the mean (Berk et al., 1996). Anesthesia decreases overall lymph flow since it reduces the systemic driving forces for lymph propulsion such as the respiration rate, blood flow and pressure, and skeletal movements (Colantuoni et al., 1984; McHale and Thornbury, 1989; Schad et al., 1976).

To transport lymph through the lymphatic system, collecting vessels possess smooth muscle and valves (Lauweryns et al., 1976; Leak and Burke, 1966). The smooth muscle exhibits spontaneous contractions in the form of peristaltic waves between lymphangions at approximately 5 mm/s (Hall et al., 1965; Hargens and Zweifach, 1977; Ohhashi et al., 1980; Olszewski and Engeset, 1980; Zawieja et al., 1993). The valves facilitate this peristaltic propulsion of lymph by allowing emptying and filling of each lymphangion—two neighboring valves are never open at the same time (Ohhashi et al., 1980)—which results in stepwise pressure changes from one lymphangion to the next. Since the spontaneous contractions can be evoked by distension (Mislin, 1976; Reddy and Staub, 1981), the presence of the valves is essential to contraction because they allow a lymphangion to distend before emptying

into the next segment. This results in a net pressure drop along the length of the collecting vessels and lymph flow ceases when rhythmic contractions stop (Ohhashi et al., 1980).

Lymphatic function is often characterized by a tissue clearance rate, which describes the removal of injected molecules or particles in terms of amount per unit time per unit tissue volume. Lymph formation can be observed in skin and mesentery by injecting an optical contrast agent such as mercury, radiolabeled particles, or fluorescently labeled macromolecules (Bollinger et al., 1981; McNeill et al., 1989; Mortimer et al., 1990; Swartz et al., 1996). This procedure is commonly termed 'microlymphangiography' and can be used to diagnose lymphatic dysfunction. Other methods for evaluating lymphatic function include measurements of solute concentration ratios between plasma and lymph (Renkin and Wiig, 1994) as well as local measurements of lymphatic capillary pressures (Bates et al., 1994; Wen et al., 1994; Zaugg-Vesti et al., 1993).

LYMPHATIC SYSTEM AND CANCER

The lymphatic system serves as the primary route for the metastasis of most cancers and the spread of tumor cells via lymphatic vessels to the regional lymph nodes is one of the most important indicators of tumor aggressiveness for the majority of human malignancies. Whereas lymphatic vessels containing clusters of tumor cells are frequently observed at the periphery of malignant tumors, it has been generally accepted that lymphatic vessels are absent from tumors themselves (Carmeliet and Jain, 2000; Folkman, 1996; Gilchrist, 1950; Lee and Tilghmanm, 1933; Leu et al., 2000; Tanigawa et al., 1981; Zeidman et al., 1955). Some early studies reported intratumoral lymphatic vessels in certain types of cancer (Evans, 1908; Reichert, 1926), but this has been interpreted mainly as co-option of preexisting lymphatic vessels by invading tumor cells. Hence, although the significance of preexisting peritumoral lymphatics as conduits for tumor cell dissemination has been well recognized (Fisher and Fisher, 1968), it has remained unclear whether tumors can stimulate lymphangiogenesis and whether tumor metastasis necessitates molecular activation of the lymphatic system (Folkman, 1996; Leu et al., 2000; Witte et al., 1997).

Several studies have failed to identify functional lymphatics within tumors (Jain, 1987; Leu et al., 2000), leading to the concept that lymphangiogenesis may not play a major role in tumor metastasis (Carmeliet and Jain, 2000). However, the absence of detectable perfusion of lymphatic vessels does not necessarily imply the absence of anatomically distinguishable lymphatic vessels from tumors. The formation of an intratumoral lymphatic network, whether fully functional in fluid transport or not, may promote metastatic tumor spread by creating increased opportunities for metastatic tumor cells to leave the primary tumor site. The presence and potential function of lymphatic vessels in tumors have remained controversial mostly due to the lack of molecular markers to reliably distinguish the lymphatic vasculature from blood vessels (Skobe and Detmar, 2000). Recently, several novel molecules have been identified that allow a more precise distinction between lymphatic and blood vascular endothelium. These include VEGFR-3 (FLT-4), the receptor for the vascular endothelial growth factors VEGF-C and VEGF-D (Veikkola et al., 2000); podoplanin, a glomerular podocyte membrane mucoprotein (Breiteneder-Geleff et al., 1999; Weninger et al., 1999); and the homeobox gene product Prox-1 that is involved in regulating development of the lymphatic system (Wigle and Oliver, 1999). Most recently, a novel hyaluronan receptor termed LYVE-1 has been shown to be restricted to lymphatic vessels in normal tissues (Banerji et al., 1999) and associated with tumors (Mandriota et al., 2001; Skobe et al., 2001a; Stacker et al., 2001).

MOLECULAR REGULATION OF TUMOR LYMPHANGIOGENESIS AND LYMPHATIC METASTASIS

Vascular endothelial growth factor-C (VEGF-C), a novel member of the VEGF family of growth factors (Joukov et al., 1996; Lee et al., 1996), was the first growth factor that was demonstrated to stimulate lymphangiogenesis in addition to angiogenesis (Jeltsch et al., 1997; Oh et al., 1997; Witzenbichler et al., 1998). The specific effects of VEGF-C on lymphangiogenesis depend on its proteolytic processing. The mature form of human VEGF-C stimulates both VEGFR-2 and VEGFR-3 and can therefore stimulate both angiogenesis and lymphangiogenesis, whereas the partially processed form preferentially binds and activates VEGFR-3 (Joukov et al., 1997) and specifically stimulates lymphangiogenesis (Skobe et al., 2001a). Structurally, VEGF-C is closely related to vascular endothelial growth factor-D (VEGF-D), which also binds to and activates VEGFR-2 and VEGFR-3 in a similar manner (Achen et al., 1998) and stimulates angiogenesis and lymphangiogenesis (Stacker et al., 2001).

A number of studies have recently reported VEGF-C expression in human tumors and its correlation to metastasis to regional lymph nodes. VEGF-C has been shown to be expressed in breast (Kurebayashi et al., 1999; Salven et al., 1998), colon (Akagi et al., 2000; Andre et al., 2000), lung (Niki et al., 2000; Ohta et al., 2000; Salven et al., 1998), thyroid (Bunone et al., 1999; Fellmer et al., 1999; Shushanov et al., 2000), gastric (Yonemura et al., 1999), and squamous cell cancers (Salven et al., 1998), mesotheliomas (Ohta et al., 1999), as well as neuroblastomas (Eggert et al., 2000), sarcomas (Salven et al., 1998), and melanomas (Salven et al., 1998). Increased expression of VEGFR-3 has been detected in lymphatic endothelium adjacent to cancer cells and in lymph nodes containing carcinoma metastases (Jussila et al., 1998; Kaipainen et al., 1995). Moreover, correlation between the VEGF-C expression and the rate of metastasis to lymph nodes has been found in breast (Kurebayashi et al., 1999), colorectal (Akagi et al., 2000), gastric (Yonemura et al., 1999), thyroid (Bunone et al., 1999; Fellmer et al., 1999), lung (Ohta et al., 2000), and prostate (Tsurusaki et al., 1999) cancers.

In addition to the abundant correlative clinical data, very recently a functional role of VEGF-C in tumor lymphangiogenesis and metastasis has been demonstrated (Mandriota et al., 2001; Skobe et al., 2001a). Overexpression of VEGF-C in genetically fluorescent human breast cancer cells transplanted onto nude mice resulted in enlargement of peritumoral lymphatic ves-

sels and in strikingly increased intratumoral lymphangiogenesis, without any obvious effects on tumor angiogenesis. Increased intratumoral lymphatic vessel density was associated with a significantly increased incidence of metastases in regional lymph nodes as well as with increased lung metastases. In fact, the extent of intratumoral lymphatic vessel density was highly correlated with the extent of lung metastases, implying an important role of the lymphatic system for the metastatic tumor spread to distant sites (Skobe et al., 2001a).

VEGF-C-induced lymphangiogenesis has also been shown to promote metastases to lymph nodes in a model of pancreatic cancer. Transgenic mice in which VEGF-C expression is driven by the rat insulin promoter (Rip) were crossed with Rip1Tag2 mice which spontaneously develop pancreatic β-cell tumors as a consequence of SV40 large T-antigen expression under the same promoter (Mandriota et al., 2001). The tumors of the Rip1Tag2 mice are locally invasive, but are neither lymphangiogenic nor metastatic (Hanahan, 1985). In the double transgenic model, VEGF-C induced lymphangiogenesis at the periphery, although not within the pancreatic β-cell tumors, which promoted the metastatic spread to regional lymph nodes (Mandriota et al., 2001). Taken together, these results provide a mechanistic explanation for the previously reported correlation of VEGF-C expression in the primary tumors with high incidence of lymph node metastases.

Another recent study demonstrated the important role of VEGF-D in tumor lymphangiogenesis and metastasis. Similar to VEGF-C, VEGF-D overexpressing epitheloid tumors induced the formation of intratumoral lymphatic vessels and promoted lymph node metastases in mice. Importantly, lymphatic spread induced by VEGF-D could be blocked with a neutralizing anti-VEGF-D antibody, suggesting inhibition of lymphangiogenesis as a useful strategy to inhibit metastatic spread of cancer (Stacker et al., 2001). While VEGF-D promoted tumor dissemination to lymph nodes, VEGF overexpression in the same experimental model did not, implying differential roles of VEGF family members in determining the route of metastases. In analogy with these findings, VEGF-C expression was detected only in node-positive human breast cancers, whereas expression of VEGF was detected in both node-positive and node-negative tumors (Kurebayashi et al., 1999).

Evidence for the existence of intratumoral lymphangiogenesis using molecular markers of the lymphatic vessels has so far been obtained only in experimental tumor models. In addition to VEGF-C overexpressing breast cancer (Skobe et al., 2001a) and VEGF-D overexpressing epitheloid tumors (Stacker et al., 2001), intratumoral lymphatic vessels were also frequently detectable within experimental cutaneous squamous cell carcinomas and VEGF-C overexpressing malignant melanomas (Skobe et al., 2001b). Furthermore, intratumoral lymphangiogenesis has been observed within human melanomas with high endogenous expression of VEGF-C transplanted onto avian chorioallantoic membrane (CAM) (Papoutsi et al., 2000). Moreover, in a breast cancer model lymphangiogenesis was induced not only within VEGF-C expressing tumors but also within nontransfected, control tumors, suggesting the production of lymphangiogenic factors other then VEGF-C in these tumors (Skobe et al., 2001a). The presence of intratumoral lymphangiogenesis in spontaneously developing human tumors and its potential prognostic significance remains to be determined.

Production of lymphangiogenic factors in tumors may promote the incidence of lymphatic metastases by increasing the number of lymphatic vessels in the vicinity of tumor cells and therefore creating increased opportunities for tumor cells to leave the primary tumor site. It is also possible, however, that the activation of lymphatics by VEGF-C, VEGF-D, or related growth factors could promote molecular interactions of tumor cells with lymphatic endothelial cells, thereby facilitating tumor cell entry into the lymphatics. Therefore, even when the tumor itself lacks lymphatic vessels, as in the VEGF-C-expressing pancreatic cancer (Mandriota et al., 2001), an increase and/or activation of peritumoral lymphatics might promote tumor metastasis. Finally, the physiology of the lymphatic system is optimally suited for the entry and transport of cells (i.e., immune cells) (Witte et al., 1997) and therefore has many advantages over the blood circulation as a transport route for a metastasizing tumor cell or embolism. The smallest lymphatic vessels are still much larger than blood capillaries and flow velocities are orders of magnitude slower. Lymph fluid is nearly identical to interstitial fluid and promotes cell viability. In contrast, tumor cells in the bloodstream experience serum toxicity, high shear stresses, and mechanical deformation leading to an extremely low success rate for metastasis (Liotta et al., 1991; Weiss and Schmid-Schönbein, 1989). The preferential metastasis via lymphatics, due to expression of lymphangiogenic factors in tumors, might therefore promote survival of disseminating tumor cells and consequently increase their metastatic efficiency. Nearly all investigations of the details of metastatic process, such as intravasation, survival, and extravasation, have focused on tumor cell behavior in the bloodstream (Liotta et al., 1991; Zetter, 1993) and there is currently a great need for clarifying the interactions between tumor cells and lymphatics and to develop a paradigm for lymphatic metastasis similar to that of hematogenous metastasis.

PERSPECTIVES

Recent findings that demonstrate the occurrence of peri- and intratumoral lymphangiogenesis in cancer and its relationship to cancer metastasis (Mandriota et al., 2001; Skobe et al., 2001a; Stacker et al., 2001) have created a basis for exploring new strategies in cancer diagnosis and therapy. However, a large amount of work is still required to evaluate the significance of tumor lymphangiogenesis in spontaneously arising human tumors and its relevance for distinct tumor types. Although correlations between expression of the lymphangiogenic factor (VEGF-C) and lymph node metastases in human tumors have been reported (Akagi et al., 2000; Bunone et al., 1999; Fellmer et al., 1999; Kurebayashi et al., 1999; Ohta et al., 2000; Tsurusaki et al., 1999; Yonemura et al., 1999), the relationship between lymphangiogenesis and metastasis in these tumors remains to be established. Preliminary evi-

dence from experimental models suggests that lymphangiogenesis might be of particular importance in tumor types that preferentially metastasize through the lymphatic system, such as breast carcinoma (Skobe et al., 2001a), melanoma (Skobe et al., 2001b), and squamous cell carcinoma (Skobe et al., unpublished data); therefore, targeting lymphangiogenesis may be therapeutically significant, in particular for certain tumor types. Clearly, targeting VEGF-C, VEGF-D, and VEGFR-3 requires further evaluation as a strategy to inhibit tumor metastases. In addition to being potential targets for inhibiting tumor metastasis, factors implicated in tumor lymphangiogenesis and specific molecules found on the activated lymphatic endothelium may prove valuable in diagnosis of particularly aggressive, metastatic cancers.

Finally, common treatments of many cancers, such as lymph node resection and radiation therapy, are frequently associated with lymphedema, a chronic condition that is a major clinical problem. Edema is characterized both by changes in the extracellular matrix and alterations of lymphatic vessels and the interplay between these two factors remains to be elucidated. It is possible that therapy aimed at promoting lymphatic regeneration may lead to an overall increase in lymphatic function in edematous tissue; however, it remains to be determined whether this can be achieved by application of lymphangiogenic growth factors alone. The continued discovery and characterization of factors that regulate lymphangiogenesis, as well as understanding the role of the extracellular matrix in lymphangiogenesis, will be essential for creating rational therapies for secondary edema associated with cancer and for the development of new therapeutic strategies for limiting cancer spread.

REFERENCES

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. 1998. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (FLT-4). Proc Natl Acad Sci USA 95:548–553.

Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y, Sugimachi K. 2000. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. Br J Cancer 83:887–891.

Andre T, Kotelevets L, Vaillant JC, Coudray AM, Weber L, Prevot S, Parc R, Gespach C, Chastre E. 2000. VEGF, VEGF-B, VEGF-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. Int J Cancer 86:174–181.

Aukland K, Nicolaysen G. 1981. Interstitial fluid volume: local regulatory mechanisms. Physiol Rev 61:556–643.

Aukland K, Reed RK. 1993. Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. Physiol Rev 73:1–78.

Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG. 1999. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol 144:789–801.

Bates DO, Levick JR, Mortimer PS. 1994. Starling pressures in the human arm and their alteration in postmastectomy oedema. J Physiol (Lond) 477:355–363.

Berk DA, Swartz MA, Leu AJ, Jain RK. 1996. Transport in lymphatic capillaries. II. Microscopic velocity measurement with fluorescence recovery after photobleaching. Am J Physiol 270:H330–337.

Bert JL, Bowen BD, Reed RK. 1988. Microvascular exchange and interstitial volume regulation in the rat: model validation. Am J Physiol 254:H384–399.

Bollinger A, Jäger K, Sgier F, Seglias J. 1981. Fluorescence microlymphangiography. Circulation 64:1195–1200.

Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, Kerjaschki D. 1999. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. Am J Pathol 154:385–394.

Bunone G, Vigneri P, Mariani L, Buto S, Collini P, Pilotti S, Pierotti MA, Bongarzone I. 1999. Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. Am J Pathol 155:1967–1976.

Carmeliet P, Jain RK. 2000. Angiogenesis in cancer and other diseases. Nature 407:249–257.

Casley-Smith JR, Florey HW. 1961. The structure of normal small lymphatics. Q J Exp Physiol 46:101.

Colantuoni A, Bertuglia S, Intaglietta M. 1984. Effects of anesthesia on the spontaneous activity of the microvasculature. Int J Microcirc Clin Exp 3:13–28.

Cornford ME, Oldendorf WH. 1993. Terminal endothelial cells of lymph capillaries as active transport structures involved in the formation of lymph in rat skin. Lymphology 26:67–78.

Daroczy J. 1988. The dermal lymphatic capillaries. Berlin, Heidelberg: Springer-Verlag.

Eggert A, Ikegaki N, Kwiatkowski J, Zhao H, Brodeur GM, Himelstein BP. 2000. High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. Clin Cancer Res 6:1900–1908.

Evans HM. 1908. On the occurrence of newly-formed lymphatic vessels in malignant growths. Johns Hopkins Med J 19:232.

Fellmer PT, Sato K, Tanaka R, Okamoto T, Kato Y, Kobayashi M, Shibuya M, Obara T. 1999. Vascular endothelial growth factor-C gene expression in papillary and follicular thyroid carcinomas. Surgery 126:1056–1061.

Fischer M, Herrig I, Costanzo U, Wen S, Schiesser M, Franzeck UK, Hoffmann U, Bollinger A. 1994. Flow velocity in single lymphatic human skin capillaries of healthy controls. Rome: 18th Annual European Conference on Microcirculation.

Fisher B, Fisher ER. 1968. Role of the lymphatic system in dissemination of tumor. In: Mayerson HS, editor. Lymph and the lymphatic system. Springfield, IL: Charles C. Thomas. p 324.

Folkman J. 1996. Angiogenesis and tumor growth. N Engl J Med 334:921.

Gerli R, Ibba L, Fruschelli C. 1990. A fibrillar elastic apparatus around human lymph capillaries. Anat Embryol 181:281–286.

Gerli R, Ibba L, Fruschelli C. 1991. Ultrastructural cytochemistry of anchoring filaments of human lymphatic capillaries and their relation to elastic fibers. Lymphology 24:105–112.

Gilchrist RK. 1950. Surgical management of advanced cancer of the breast. Arch Surg 61:913–929.

Guyton AC. 1965. Pressure-volume relationships in the interstitial spaces. Invest Ophthalmol 4:1075–1084.

Hall JG, Morris B, Woolley G. 1965. Intrinsic rhythmic propulsion of lymph in the unanesthetized sheep. J Physiol Lond 180:336–349.

Hanahan D. 1985. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature 315:115–22.

Hargens AR, Zweifach BW. 1977. Contractile stimuli in collecting lymph vessels. Am J Physiol 233:H57–H65.

Hogan RD, Unthank JL. 1986. The initial lymphatics as sensors of interstitial fluid volume. Microvasc Res 31:317–324.

Ikomi F, Schmid-Schönbein GW. 1996. Lymph pump mechanics in the rabbit hind leg. Am J Physiol 271:H173–183.

Jain RK. 1987. Transport of molecules in the tumor interstitium: a review. Cancer Res 47:3039–3051.

Jeltsch M, Kaipainen A, Joukov V, Kukk E, Lymbousssaki A, Lakso M, Alitalo K. 1997. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 276:1423–1425.

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. 1996. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:1751.

Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson WL, Cao Y, Saksela O, Kalkkinen N, Alitalo K. 1997. Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 16:3898–3911.

Jussila L, Valtola R, Partanen TA, Salven P, Heikkila P, Matikainen MT, Renkonen R, Kaipainen A, Detmar M, Tschachler E, Alitalo R, Alitalo K. 1998. Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. Cancer Res 58:1599–1604.

Kaipainen A, Korhonen J, Mustonen T, van HV, Fang GH, Dumont D, Breitman M, Alitalo K. 1995. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 92:3566–3570.

Kurebayashi J, Otsuki T, Kunisue H, Mikami Y, Tanaka K, Yamamoto S, Sonoo H. 1999. Expression of vascular endothelial

growth factor (VEGF) family members in breast cancer. Jpn J Cancer Res 90:977–981.
Lauweryns JM, Baert J, de Loecker W. 1976. Fine filaments in lymphatic endothelial cells. J Cell Biol 68:163–167.
Leak LV. 1970. Electron microscopic observations on lymphatic capillaries and the structural components of the connective tissue-lymph interface. Microvasc Res 2:361–391.
Leak LV. 1976. The structure of lymphatic capillaries in lymph formation. Fed Proc 35:1863–1871.
Leak LV, Burke JF. 1966. Fine structure of the lymphatic capillary and the adjoining connective tissue area. Am J Anat 118:785–810.
Lee FC, Tilghmanm RC. 1933. Lymph vessels in rabbit carcinoma, with a note on the normal lymph vessel structure of the testis. Arch Surg 26:602–616.
Lee J, Gray A, Yuan J, Luoh SM, Avraham H, Wood WI. 1996. Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. Proc Natl Acad Sci USA 93:1988–1992.
Leu AJ, Berk DA, Lymboussaki A, Alitalo K, Jain RK. 2000. Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. Cancer Res 60:4324–4327.
Liotta LA, Steeg PS, Stetler-Stevenson WG. 1991. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 64:327–336.
Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G, Pepper MS. 2001. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. EMBO J 20:672–682.
Marchetti C, Poggi P, Piacentini C, Calligaro A. 1991. Scanning and transmission electron microscopy of rabbit heart lymphatic capillaries. Lymphology 24:26–31.
McGeown JG, McHale NG, Thornbury KD. 1988. Effects of varying patterns of external compression on lymph flow in the hindlimb of the anaesthetized sheep. J Physiol Lond 397:449–457.
McHale NG, Thornbury KD. 1989. The effect of anesthetics on lymphatic contractility. Microvasc Res 37:70–76.
McNeill GC, Witte MH, Witte CL, Williams WH, Hall JN, Patton DD, Pond GD, Woolfenden JM. 1989. Whole-body lymphangioscintigraphy: preferred method of initial assessment of the peripheral lymphatic system. Radiology 172:495–502.
Mislin H. 1976. Active contractility of the lymphangion and coordination of lymphangion chains. Experimentia Basel 32:820–822.
Mortimer PS, Simmonds R, Rezvani M, Robbins M, Hopewell JW, Ryan TJ. 1990. The measurement of skin lymph flow by isotope clearance—reliability, reproducibility, injection dynamics, and the effect of massage. J Invest Dermatol 95:677–682.
Negrini D, Ballard ST, Benoit JN. 1994. Contribution of lymphatic myogenic activity and respiratory movements to pleural lymph flow. J Appl Physiol 76:2267–2274.
Negrini D, Passi A, DeLuca G, Miserocchi G. 1996. Pulmonary interstitial pressure and proteoglycans during development of pulmonary edema. Am J Physiol 270:H2000–2007.
Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y, Hirohashi S. 2000. Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. Clin Cancer Res 6:2431–2439.
Oh SJ, Jeltsch MM, Birkenhager R, McCarthy JE, Weich HA, Christ B, Alitalo K, Wilting J. 1997. VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. Dev Biol 188:96–109.
Ohhashi T, Azuma T, Sakaguchi M. 1980. Active and passive mechanical characteristics of bovine mesenteric lymphatics. Am J Physiol 239:H88–95.
Ohta Y, Shridhar V, Bright RK, Kalemkerian GP, Du W, Carbone M, Watanabe Y, Pass HI. 1999. VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. Br J Cancer 81:54–61.
Ohta Y, Nozawa H, Tanaka Y, Oda M, Watanabe Y. 2000. Increased vascular endothelial growth factor and vascular endothelial growth factor-c and decreased nm23 expression associated with microdissemination in the lymph nodes in stage I non-small cell lung cancer. J Thorac Cardiovasc Surg 119:804–813.
Olszewski WL, Engeset A. 1980. Intrinsic contractility of prenodal lymph vessels and lymph flow in human leg. Am J Physiol 239: H775–H783.
Olszewski WL, Engeset A, Jaeger M, Sokolowski J, Theodorsen L. 1977. Flow and composition of leg lymph in normal men during venous stasis, muscular activity and local hyperthermia. Acta Physiol Scand 99:149–155.
Papoutsi M, Siemeister G, Weindel K, Tomarev SI, Kurz H, Schachtele C, Martiny-Baron G, Christ B, Marme D, Wilting J. 2000. Active interaction of human A375 melanoma cells with the lymphatics in vivo. Histochem Cell Biol 114:373–385.
Parsons RJ, McMaster PD. 1938. The effect of the pulse upon the formation and flow of lymph. J Exp Med 68:353–376.
Pullinger BD, Florey HW. 1935. Some observations on the structure and functions of lymphatics: their behavior in local edema. Br J Exp Pathol 16:49.
Reddy NP, Staub NC. 1981. Intrinsic propulsive activity of thoracic duct perfused in anesthetized dogs. Microvasc Res 21:183–192.
Reichert FL. 1926. The regeneration of the lymphatics. Arch Surg 13:871–881.
Renkin EM, Wiig H. 1994. Limits to steady-state lymph flow rates derived from plasma-to-tissue uptake measurements. Microvasc Res 47:318–328.
Salven P, Lymboussaki A, Heikkila P, Jaaskela-Saari H, Enholm B, Aase K, von Euler G, Eriksson U, Alitalo K, Joensuu H. 1998. Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. Am J Pathol 153:103–108.
Schad H, Brechtelsbauer H, Kramer K. 1976. Effect of anesthesia (Halothane) on thoracic duct lymph flow and lymph composition in dogs. Ergeb Angiol 12:185–189.
Schad H, Flowaczny H, Brechtelsbauer H, Birkenfeld G. 1978. The significance of respiration for thoracic duct flow in relation to other driving forces of lymph flow. Pfluegers Arch 378:121–125.
Schmid-Schönbein GW. 1990a. Mechanisms causing initial lymphatics to expand and compress to promote lymph flow. Arch Histol Cytol 53 Suppl:107–114.
Schmid-Schönbein GW. 1990b. Microlymphatics and lymph flow. Physiol Rev 70:987–1028.
Shushanov S, Bronstein M, Adelaide J, Jussila L, Tchipysheva T, Jacquemier J, Stavrovskaya A, Birnbaum D, Karamysheva A. 2000. VEGF-C and VEGFR3 expression in human thyroid pathologies. Int J Cancer 86:47–52.
Skobe M, Detmar M. 2000. Structure, function and molecular control of the skin lymphatic system. Rev J Invest Dermatol Symp Proceed 5:14–19.
Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M. 2001a. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 7:192–198.
Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, Detmar M. 2001b. Concurrent induction of lymphangiogenesis, angiogenesis and macrophage recruitment by VEGF-C in melanoma. Am J Pathol, in press.
Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG. 2001. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 7:186–191.
Swartz MA, Berk DA, Jain RK. 1996. Transport in lymphatic capillaries. I. Macroscopic measurement using residence time distribution analysis. Am J Physiol 270:H324–329.
Tanigawa N, Kanazawa T, Satomura K, Hikasa Y, Hashida M, Muranishi S, Sezaki H. 1981. Experimental study on lymphatic vascular changes in the development of cancer. Lymphology 14:149–154.
Taylor AE, Gibson WH, Granger HJ, Guyton AC. 1973. The interaction between intracapillary and tissue forces in the overall regulation of interstitial fluid volume. Lymphology 6:192–208.
Tsurusaki T, Kanda S, Sakai H, Kanetake H, Saito Y, Alitalo K, Koji T. 1999. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer 80:309–313.
Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo K. 2000. Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res 60:203–212.
Weiss L, Schmid-Schönbein GW. 1989. Biomechanical interactions of cancer cells with the microvasculature during metastasis. Cell Biophys 14:187–215.
Wen S, Dorffler-Melly J, Herrig I, Schiesser M, Franzeck UK, Bollinger A. 1994. Fluctuation of skin lymphatic capillary pressure in controls and in patients with primary lymphedema. Int J Microcirc Clin Exp 14:139–143.
Weninger W, Partanen TA, Breiteneder-Geleff S, Mayer C, Kowalski H, Mildner M, Pammer J, Sturzl M, Kerjaschki D, Alitalo K, Tschachler E. 1999. Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells. Lab Invest 79:243–251.

Wigle JT, Oliver G. 1999. Prox1 function is required for the development of the murine lymphatic system. Cell 98:769–778.
Witte MH, Way DL, Witte CL, Bernas M. 1997. Lymphangiogenesis: mechanisms, significance and clinical implications. EXS 79:65–112.
Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu JS, Isner JM. 1998. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. Am J Pathol 153:381–394.
Yoffey JM, Courtice FC. 1970. Lymphatics, lymph and the lymphomyeloid complex. London: Academic Press.
Yonemura Y, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K, Sasaki T. 1999. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. Clin Cancer Res 5:1823–1829.
Zaugg-Vesti B, Dorffler-Melly J, Spiegel M, Wen S, Franzeck UK, Bollinger A. 1993. Lymphatic capillary pressure in patients with primary lymphedema. Microvasc Res 46:128–134.
Zawieja DC, Davis KL, Schuster R, Hinds WM, Granger HJ. 1993. Distribution, propagation, and coordination of contractile activity in lymphatics. Am J Physiol 264:H1283–1291.
Zeidman I, Copeland BE, Warren S. 1955. Experimental studies on the spread of cancer in the lymphatic system. II. Absence of a lymphatic supply in carcinomas. Cancer 8:123–127.
Zetter BR. 1993. Adhesion molecules in tumor metastasis. Semin Cancer Res 4:219–229.

Lymphatic Vessel Activation in Cancer

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ABSTRACT: Metastasis of most cancers occurs primarily through the lymphatic system, and the extent of lymph node involvement is the most important prognostic indicator. While the importance of the lymphatic system as a pathway for metastasis has been well recognized, there is very little information available about the mechanisms by which tumor cells interact with the lymphatics. Recently, production of the lymphangiogenic factor VEGF-C has been detected in tumors, and the significance of VEGF-C-mediated lymphangiogenesis for tumor metastasis has been demonstrated. Increased lymphatic vessel density has been found associated with certain tumors. The mechanisms by which tumor cells gain access to and enter lymphatic vessels are critical issues that need to be addressed in the future. In contrast to the prevailing view that has assigned to the lymphatic system a passive role in the metastatic process, our results indicate the importance of lymphatic vessel activation in tumor dissemination.

KEYWORDS: lymphangiogenesis; lymphatic endothelium; cancer metastasis; VEGF-C; VEGF receptor

INTRODUCTION

The metastatic spread of tumor cells is a major cause of death in cancer patients. The lymphatic system is the primary pathway of metastasis for most human cancers, and the extent of lymph node involvement is a key prognostic factor for the patient's outcome. In spite of this, most experimental work addressing tumor dissemination has focused on hematogenous spread.[1,2] In fact, the ability of tumor cells to induce angiogenesis is considered a prerequisite for tumor growth, invasion, and successful metastasis, and the angiogenic switch is recognized as one of the key events in tumorigenesis. In contrast, very little effort has been directed towards understanding the molecular regulation of lymphatic vessel formation and function, and the major

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issues regarding the role of lymphatic vessels in tumor growth and metastasis remain unresolved.[3] We are now beginning to gain insight into the function of lymphatic vessels in tumor progression, and it remains to be determined whether lymphangiogenesis and/or activation of the lymphatic system is an integral part of tumorigenesis in humans.

The capacity of lymphatic vessels to regenerate was first observed nearly a century ago.[4–6] Pronounced lymphatic growth has been detected in wound healing and inflammation, and some studies have reported an increase of lymphatic vessel density associated with certain tumors.[3,6,7] These findings did not receive much attention, ~~chiefly~~ for two reasons. First, the credibility of the findings has been questioned, due to the lack of markers that would have allowed reliable distinction of lymphatic from blood vasculature. Second, the lymphatic system has traditionally been assigned a passive role in cancer metastasis; therefore, any significance of these findings has been questioned. Consequently, while the importance of the lymphatic system as a pathway for metastasis has been well recognized, there is very little information available about the mechanisms by which tumor cells interact with the lymphatics.

The function of lymphatic vessels in cancer remains an area of controversy. One debate revolves around the question of whether tumor lymphangiogenesis exists. Very few studies have addressed this issue altogether, and only recently has evidence been obtained for tumor lymphangiogenesis by using novel molecular markers of lymphatics. However, it remains an open question whether lymphangiogenesis is a common event during tumorigenesis and whether it is restricted only to certain types of cancer and/or tumor stages. Furthermore, the overall significance of lymphangiogenesis for tumor progression in autochthonous human tumors still needs to be examined.

Another hotly debated issue concerns the presence and biological significance of intratumoral lymphatic vessels. Some early studies reported intratumoral lymphatic vessels in certain types of cancer.[5–7] However, this has been interpreted mainly as a co-option of pre-existing lymphatic vessels by invading tumor cells, and it has been proposed that lymphatic vessels are absent from most tumors.[8–12] Recently, this view has begun to change, as more specific markers of lymphatic vessels have become available.[13] Nevertheless, the issue is far from being resolved, and the functional significance of intratumoral lymphatics is the subject of particularly vigorous debate.[14] Finally, the prevailing view has been that the lymphatic system plays a passive role in the metastatic process, although the evidence ~~in support of~~ this concept is lacking. ~~Whether~~ lymphatic vasculature has an active role in promoting tumor cell metastasis ~~is a subject of ongoing investigation.~~

ROLE OF VEGF-C IN TUMOR LYMPHANGIOGENESIS AND METASTASIS

Vascular endothelial growth factor-C (VEGF-C), a novel member of the VEGF family of growth factors,[15,16] was the first lymphangiogenic factor identified. There is ample evidence for expression of VEGF-C in human tumors. VEGF-C has been shown to be expressed in breast,[17,18] colon,[19,20] lung,[18,21,22] thyroid,[23–25] gastric,[26] and squamous cell cancers[18]; in mesotheliomas[27] as well as neuroblastomas[27,28] and in sarcomas and melanomas.[18] Moreover, a correlation between VEGF-C expression and rate of metastasis to lymph nodes has been found in breast,[17] colorectal,[19] gastric,[26] thyroid,[23,24] lung,[26] and prostate[29] cancers. Expression of the lymphangiogenic factor VEGF-C in tumors has suggested, for the first time, an active interaction between tumor cells and lymphatics.

The question as to the significance of VEGF-C expression for tumor progression remains unresolved. To address this question and assess the functional importance of lymphangiogenesis for cancer metastasis, we have engineered genetically fluorescent MDA-MB-435/GFP human breast cancer cells to overexpress VEGF-C.[30] Using this orthotopic breast cancer model in immunosuppressed mice, we demonstrated that VEGF-C increased peritumoral and intratumoral lymphatic vessel density. Overexpression of VEGF-C also resulted in significant enlargement of peritumoral, but not intratumoral lymphatic vessels. Whereas the overall lymphatic vessel density was fairly consistent between the tumors, considerable regional heterogeneity within individual tumors was found. This was not a result of regional differences in VEGF-C expression, and may instead reflect local variations of the tumor microenvironment, such as differences in extracellular matrix composition and/or mechanical forces. Because integration of lymphatic vessels with the interstitium is critical for lymphatic function,[3,4,31] the extracellular microenvironment is also likely to be critical for lymphangiogenesis. Importantly, VEGF-C-mediated increase of tumor lymphangiogenesis resulted in enhanced tumor metastases to regional lymph nodes and lungs. The degree of tumor metastases was highly correlated with the intratumoral lymphatic vessel density as well as with the depth of lymphatic vessel invasion into the tumors.[30]

Our findings demonstrate a causal role for lymphangiogenesis in tumor metastasis, and provide a mechanistic explanation for the reported correlation of VEGF-C expression in primary tumors with high incidence of metastases in patients. VEGF-C may promote metastasis by increasing the number of lymphatic vessels in the vicinity of tumor cells, thereby creating increased opportunities for tumor cells to leave the primary tumor site. It is also a possibility that the activation of lymphatics by VEGF-C could induce secretion of chemokines and similar factors by the lymphatic endothelium, thereby

attracting tumor cells and facilitating their entry into lymphatics. Therefore, even when the tumor itself lacks lymphatic vessels, as in the VEGF-C-expressing pancreatic cancer,[32] an activation of peritumoral lymphatics could explain increased tumor metastasis.

What mechanism accounts for the increase of lung metastases observed in the VEGF-C-expressing breast cancer model? The accelerated growth of tumor cells in the lung is an unlikely answer, in that VEGF-C overexpression did not confer a growth advantage to tumor cells at the primary tumor site, lymph nodes, or *in vitro*, although this cannot be fully excluded. Furthermore, since VEGF-C did not increase angiogenesis in this tumor model, it is unlikely that increased access to blood vasculature accounted for the increase in metastases. One apparent possibility is that the increased ~~incidence of lung colonization by tumor cells~~ resulted from an increased ~~rate of metastasis~~ via the lymphatics. From the lymph node, tumor dissemination can occur via efferent lymphatics or through lymphatic-venous communications within the nodes, and subsequently via the blood stream.[33] The lymphatic system is optimally adapted for the entry and transport of cells, and therefore has many advantages over the blood circulation as a transport route for a metastasizing tumor cell or embolism.[3,5] The smallest lymphatic vessels are still much larger than blood capillaries, and flow velocities are orders of magnitude slower. Lymph fluid is similar to interstitial fluid and promotes cell viability. In contrast, tumor cells in the bloodstream experience serum toxicity, high shear stresses, and mechanical deformation, leading to an extremely low success rate of metastasis.[2,34] The preferential metastasis via lymphatics, due to expression of lymphangiogenic factors in tumors for example, might therefore promote survival of disseminating tumor cells and consequently increase their metastatic efficiency.

That distant metastases may increase as a consequence of increased lymphatic spread does not imply, as recently suggested,[35] that the lymphatic pathway is the exclusive pathway for metastasis in a particular tumor model. For example, in the MDA-435 breast cancer model, 100% of the mice presented with lung metastases, whereas approximately 50% of the mice had lymph node metastases at the given time point.[30] This indicates that at least a fraction of tumor metastases in the lung were blood-borne. Quantitative analysis revealed, however, that the total lung area containing metastases was greater in mice bearing VEGF-C-overexpressing tumors, suggesting a ~~substantial~~ contribution of the lymphatic pathway to the overall lung tumor burden. While many types of tumor cells are capable of spreading through both blood and lymphatic vasculature, the relative contributions of the lymphatic and hematogenous pathways for dissemination of a particular tumor are difficult to assess. In conformity with the above concept, the fact that blocking VEGFR-3 did not reduce lung metastases in a lung tumor model[36]

suggests that the spread to lungs of the given tumor is predominantly hematogenous and that VEGFR-3 signaling was not implicated.

ROLE OF VEGF-C IN TUMOR ANGIOGENESIS AND MACROPHAGE RECRUITMENT

Whereas overexpression of VEGF-C in the breast cancer model selectively induced tumor lymphangiogenesis,[30] studies of human melanoma xenotransplants revealed the induction of both lymphangiogenesis and angiogenesis by tumor-derived VEGF-C.[37] These distinct biological effects can be explained by the differential proteolytic processing of VEGF-C in the two tumor types. In breast cancer, the major VEGF-C form detected was the secreted 31-kDa protein that activates VEGFR-3 expressed mainly by the lymphatic vessels.[38–40] Accordingly, phosphorylation of VEGFR-3, but not VEGFR-2, was markedly increased in these tumors, which resulted in selective induction of lymphangiogenesis. In contrast, melanomas produced mainly the mature 21-kDa form of VEGF-C,[37] which activates VEGFR-3 and VEGFR-2 on lymphatic and blood vasculature respectively,[38] resulting in increased angiogenesis in addition to lymphangiogenesis. These results demonstrate that the biological effects of VEGF-C in tumors are critically dependent on the proteolytic processing. Processing to the 21-kDa protein was observed only *in vivo*, indicating a crucial role of host cells in regulating this process.[37]

Lymphatic vessels, serving as a pathway for the trafficking of leukocytes, are also important components of the immune system. Recent evidence suggests that, in addition to its effects on the vasculature, VEGF-C might also have a more direct impact on immune functions.[37] VEGFR-3 expression was detected on macrophages *in vitro* and *in vivo*, and VEGF-C-induced macrophage chemotaxis in a dose-dependent manner. In agreement with these results, expression of VEGFR-3 has been reported in certain hematopoietic and leukemia cells.[41] VEGF-C also increased peritumoral macrophage densities in melanoma xenografts.[37] These findings identify a novel function of VEGF-C as an immunomodulator and suggest its possible proinflammatory activities.

TUMOR LYMPHANGIOGENESIS: FACTS AND CONTROVERSIES

The prevailing belief to date has been that lymphangiogenesis does not take place in cancer and that lymphatic vessels are absent from most tumors.[11,12,14,42] Very few studies, however, have addressed these issues, and comprehensive evidence in favor of this widely accepted view is lacking.

TABLE 1. Evidence for presence of intratumoral lymphatics

Tumor Type	Marker	Reference
Experimental tumors		
Melanoma/CAM	Prox-1	Papoutsi *et al.*[47] (2000)
Pancreatic cancer/CAM	Prox-1	Papoutsi *et al.*[48] (2001)
MDA-MB-435 breast cancer control and VEGF-C	LYVE-1/VEGFR-3	Skobe *et al.*[30] (2001)
MeWo melanoma/VEGF-C-transfected	LYVE-1/VEGFR-3	Skobe *et al.*[37] (2001)
293EBNA/VEGF-D	LYVE-1	Stacker *et al.*[48] (2001)
MCF-7/VEGF-C breast cancer	LYVE-1/VEGFR-3	Karpanen *et al.*[49] (2001)
MCF-7/VEGF-C breast cancer	LYVE-1/VEGFR-3	Mattila *et al.*[50] (2002)
A431/SCC	LYVE-1/Prox-1	Wigle *et al.*[35] (2002)
Human tumors		
Breast cancer	Podoplanin	Schoppmann *et al.*[36] (2001)
Head and neck SCC	LYVE-1	Beasley *et al.*[51] (2002)

CHANGE TO:
53 → 48
48 → 49
49 → 50
54 → 51
55 → 52
56 → 53
57 → 54

Identification of molecular markers of lymphatic vessels has now made it possible to re-examine the established views. What is the evidence for absence or presence of lymphatics in tumors? Using the novel molecular markers, lymphatic channels have so far been observed in eight different experimental tumor models, in autochthonous human breast cancers, and in head and neck squamous cell carcinomas (TABLE 1). No evidence for intratumoral lymphatics has been found in human melanomas, cervical, ovarian, and liver carcinomas, or in the experimental models of pancreatic cancer and melanoma (TABLE 2). Future studies are required to determine whether intratumoral lymphatics are restricted only to certain types of cancer and whether their presence in tumors has any prognostic significance.

Most recently, the significance of intratumoral lymphatics for tumor dissemination has been called into question.[14,43] Lymphatic vessels that were identified in tumors using molecular markers of lymphatics could not be detected using lymphangiography, a technique that involves injection of labeled macromolecules into the interstitium for uptake into lymphatic capillaries. Based on these results, it has been concluded that tumors contained no functional lymphatics and that tumor cells can therefore not utilize intratumoral lymphatics for transport to the lymph nodes.[43] However, functional impairment of lymphatic vessels with respect to fluid and macromolecular transport is not the only possible explanation for the absence of detectable perfusion of lymphatics in tumors. Fluid and macromolecules travel through tissues according to hydrostatic and oncotic pressure gradients, following the pathways of least resistance to transport.[44] Elastic fibers, for example, represent a low

TABLE 2. Evidence for absence of intratumoral lymphatics

Tumor Type	Marker	Reference
Experimental tumors		
Rip1Tag2 x RipVEGF-C transgenic mice	LYVE-1/VEGFR-3	Mandriota *et al.*[32] (2001)
MeWo melanoma	LYVE-1/VEGFR-3	Skobe *et al.*[37] (2001)
Human tumors		
Cervical cancer	Podoplanin	Birner *et al.*[50] (2001)
Ovarian cancer	Podoplanin	Birner *et al.*[51] (2000)
Melanoma	CD31+ / PAL-E-	De Waal *et al.*[58] (1997)
Uveal melanoma	CD31+ / PAL-E-	Clarijs *et al.*[59] (2001)
Liver cancer	LYVE-1/Prox-1	Carreira *et al.*[52] (2001)

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resistance path for interstitial transport of fluid and are thus regarded to as pre-lymphatic pathways.[45] In normal tissues, extracellular matrix fibers are ideally arranged for directing fluid into the vessels.[45] In tumors, however, the extracellular matrix composition and organization are commonly altered and it is plausible that the fluid channels in tumor stroma are not directing fluid into the tumor lymphatics in such an organized manner. Furthermore, elevated interstitial fluid pressure has been reported in tumors,[46] resulting in steep hydrostatic pressure gradients at the tumor edge that may force fluid primarily out of the tumor and not laterally into tumor lymphatics. While the impairment of lymphatic ability to take up fluid could explain the absence of lymphatic vessel perfusion in tumors, it is yet to be determined which of the hypotheses provides the correct answer.

Importantly, whereas the functional state of tumor lymphatic vessels with respect to the efficient uptake of fluids and macromolecules is of great importance for overall tumor physiology and drug delivery,[46] it may not be crucial for tumor dissemination. Because of the lack of detectable accumulation of an interstitially injected tracer in tumor lymphatics, it has been interpreted that these can not be utilized by tumor cells for migration to lymph nodes.[14,43] Such a conclusion is based on the assumption that the transport of fluid and cells in tissues and their uptake into the lymphatics is governed by the same principles. This is unlikely, as cell migration in tissues is a tightly controlled process involving a defined set of cell interactions with their microenvironment, such as responsiveness to soluble factors, attachment to specific components of the extracellular matrix, and localized proteolysis. In contrast, the major forces controlling uptake of fluid and macromolecules into lymphatics are pressure gradients in tissue.[44] Whether these forces have any effect on cell transport into the lymphatics remains an open question. Hence, the formation of an intratumoral lymphatic network, whether fully functional in fluid uptake or not, may still promote metastatic tumor spread

by creating increased opportunities for metastatic tumor cells to leave the primary tumor site. Moreover, lymphatic vasculature may become activated in tumors and increase tumor cell propensity to metastasize. For example, activation of lymphatics by VEGF-C or VEGF-D could promote production of chemoattractants by lymphatic endothelial cells and thereby facilitate tumor cell entry into the lymphatics.

In conclusion, despite the intense discussion revolving around the presence and significance of intratumoral lymphatics, these might not be the most relevant questions to be answered. It is evident that tumor cells can utilize peritumoral lymphatics to spread; therefore, intratumoral lymphatics should be regarded as an additional pathway rather than a necessity for metastasis. A more fundamental issue to be addressed is the mechanism by which tumor cells enter lymphatics, regardless of their location. Our recent data indicate an active role of lymphatic endothelium in tumor metastasis, the underlying mechanisms of which are a subject of ongoing investigation.

REFERENCES

1. ZETTER, B.R. 1993. Adhesion molecules in tumor metastasis. Sem. Cancer Res. **4:** 219–229.
2. LIOTTA, L.A., P.S. STEEG & W.G. STETLER-STEVENSON. 1991. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell **64:** 327–336.
3. SWARTZ, M.A. & M. SKOBE. 2001. Lymphatic function, lymphangiogenesis, and cancer metastasis. Microsc. Res. Tech. **55:** 92–99.
4. PULLINGER, B.D. & H.W.FLOREY. 1935. Some observations on the structure and functions of lymphatics: their behavior in local edema. Br. J. Exp. Pathol. **16:** 49.
5. WITTE, M.H., D.L. WAY, C.L.WITTE, *et al.* 1997. Lymphangiogenesis: mechanisms, significance and clinical implications. Exs **79:** 65–112.
6. REICHERT, F.L. 1926. The regeneration of the lymphatics. Arch. Surg. **13:** 871–881.
7. EVANS, H.M. 1908. On the occurence of newly formed lymphatic vessels in malignant growths. Johns Hopkins Med. J. **19:** 232.
8. LEE, F.C. & R.C. TILGHMANN. 1933. Lymph vessels in rabbit carcinoma, with a note on the normal lymph vessel structure of the testis. Arch. Surg. **26:** 602–616.
9. GILCHRIST, R.K. 1950. Surgical management of advanced cancer of the breast. Arch. Surg. **61:** 913–929.
10. ZEIDMAN, I., B.E. COPELAND & S. WARREN. 1955. Experimental studies on the spread of cancer in the lymphatic system. II. Absence of a lymphatic supply in carcinomas. Cancer **8:** 123–127.
11. FOLKMAN, J. 1996. Angiogenesis and tumor growth. N. Engl. J. Med. **334:** 921.

12. LEU, A.J., D.A. BERK, A. LYMBOUSSAKI, *et al.* 2000. Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. Cancer Res **60:** 4324–4327.
13. SLEEMAN, J.P., J. KRISHNAN, V. KIRKIN, *et al.* 2001. Markers for the lymphatic endothelium: in search of the holy grail? Microsc. Res. Tech. **55:** 61–69.
14. JAIN, R.K. & B.T. FENTON. 2002. Intratumoral lymphatic vessels: a case of mistaken identity or malfunction? J. Natl. Cancer Inst. **94:** 417–421.
15. JOUKOV, V., K. PAJUSOLA, A. KAIPAINEN, *et al.* 1996. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J. **15:** 290–298.
16. LEE, J., A. GRAY, J. YUAN, *et al.* 1996. Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. Proc. Natl. Acad. Sci. USA **93:** 1988–1992.
17. KUREBAYASHI, J., T. OTSUKI, H. KUNISUE, *et al.* 1999. Expression of vascular endothelial growth factor (VEGF) family members in breast cancer. Jpn. J. Cancer Res. **90:** 977–981.
18. SALVEN, P., A. LYMBOUSSAKI, P. HEIKKILA, *et al.* 1998. Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. Am. J. Pathol. **153:** 103–108.
19. AKAGI, K., Y. IKEDA, M. MIYAZAKI, *et al.* 2000. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. Br. J. Cancer **83:** 887–891.
20. ANDRE, T., L. KOTELEVETS, J.C. VAILLANT, *et al.* 2000. Vegf, Vegf-B, Vegf-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. Int. J. Cancer **86:** 174–181.
21. NIKI, T., S. IBA, M. TOKUNOU, *et al.* 2000. Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma./Clin. Cancer Res. **6:** 2431–2439.
22. OHTA, Y., V. SHRIDHAR, R.K. BRIGHT, *et al.* 1999. VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. Br. J. Cancer **81:** 54–61.
23. BUNONE, G., P. VIGNERI, L. MARIANI, *et al.* 1999. Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. Am. J. Pathol. **155:** 1967–1976.
24. FELLMER, P.T., K. SATO, R. TANAKA, *et al.* 1999. Vascular endothelial growth factor-C gene expression in papillary and follicular thyroid carcinomas. Surgery **126:** 1056–1061.
25. SHUSHANOV, S., M. BRONSTEIN, J. ADELAIDE, *et al.* 2000. VEGF-C and VEGFR3 expression in human thyroid pathologies. Int. J. Cancer **86:** 47–52.
26. YONEMURA, Y., Y. ENDO, H. FUJITA, *et al.* 1999. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. Clin. Cancer Res. **5:** 1823–1829.
27. EGGERT, A., N. IKEGAKI, J. KWIATKOWSKI, *et al.* 2000. High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. Clin. Cancer Res. **6:** 1900–1908.
28. OHTA, Y., H. NOZAWA, Y. TANAKA, *et al.* 2000. Increased vascular endothelial growth factor and vascular endothelial growth factor-c and decreased nm23 expression associated with microdissemination in the lymph nodes in stage I non-small cell lung cancer. J. Thorac. Cardiovasc. Surg. **119:** 804–813.

29. TSURUSAKI, T., S. KANDA, H. SAKAI, *et al.* 1999. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br. J. Cancer **80:** 309–313.
30. SKOBE, M., T. HAWIGHORST, D.G. JACKSON, *et al.* 2001. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat. Med. **7:** 192–198.
31. LEAK, L.V. 1970. Electron microscopic observations on lymphatic capillaries and the structural components of the connective tissue-lymph interface. Microvasc. Res. **2:** 361–391.
32. MANDRIOTA, S.J., L. JUSSILA, M. JELTSCH, *et al.* 2001. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. EMBO J. **20:** 672–682.
33. FISHER, B. & E.R. FISHER. 1968. Role of the lymphatic system in dissemination of tumor. *In* Lymph and the Lymphatic System. H.S. Mayerson, Ed.: 324. Charles C. Thomas. Springfield, IL.
34. WEISS, L. & G.W. SCHMID-SCHÖNBEIN. 1989. Biomechanical interactions of cancer cells with the microvasculature during metastasis. Cell Biophys. **14:** 187–215.
35. JAIN, R.K. & T.P. PADERA. 2002. Prevention and treatment of lymphatic metastasis by antilymphangiogenic therapy. J. Natl. Cancer Inst. **94:** 785–787.
36. HE, Y., K. KOZAKI, T. KARPANEN, *et al.* 2002. Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J. Natl. Cancer Inst. **94:** 819–825.
37. SKOBE, M., L.M. HAMBERG, T. HAWIGHORST, *et al.* 2001. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. Am. J. Pathol. **159:** 893–903.
38. JOUKOV, V., T. SORSA, V. KUMAR, *et al.* 1997. Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J. **16:** 3898–3911.
39. KAIPAINEN, A., J. KORHONEN, T. MUSTONEN, *et al.* 1995. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc. Natl. Acad. Sci. USA **92:** 3566–3570.
40. JUSSILA, L., R.VALTOLA, T.A. PARTANEN, *et al.* 1998. Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. Cancer Res. **58:** 1599–1604.
41. FIELDER, W., U. GRAEVEN, S. ERGUN, *et al.* 1997. Expression of FLT4 and its ligand VEGF-C in acute myeloid leukemia. Leukemia **11:** 1234–1237.
42. CARMELIET, P. & R.K. JAIN. 2000. Angiogenesis in cancer and other diseases. Nature **407:** 249–257.
43. PADERA, T.P., A. KADAMBI, E.DI TOMASO, *et al.* 2002. Lymphatic metastasis in the absence of functional intratumor lymphatics. Science **296:** 1883–1886.
44. SCHMID-SCHÖNBEIN, G.W. 1990. Microlymphatics and lymph flow. Physiol. Rev. **70:** 987–1028.
45. RYAN, T.J. 1989. Structure and function of lymphatics. J. Invest. Dermatol. **93:** 18S–24S.
46. JAIN, R.K. 1990. Vascular and interstitial barriers to delivery of therapeutic agents in tumors. Cancer Metast. Rev. **9:** 253–266.

47. PAPOUTSI, M., G. SIEMEISTER, K. WEINDEL, *et al.* 2000. Active interaction of human A375 melanoma cells with the lymphatics in vivo. Histochem. Cell Biol. **114:** 373–385.
48. PAPOUTSI, M. *et al.* 2001.
49. STACKER, S.A., C. CAESAR, M.E. BALDWIN, *et al.* 2001. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat. Med. **7:** 186–191.
50. KARPANEN, T., M. EGEBLAD, M.J. KARKKAINEN, *et al.* 2001. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res. **61:** 1786–1790.
51. MATTILA, ET AL. 2002.
52. WIGLE, *et al.* 2002.
53. SHOPPMANN, *et al.* 2001.
54. BEASLEY, *et al.* 2002.
55. BIRNER, P., M. SCHINDL, A. OBERMAIR, *et al.* 2001. Lymphatic microvessel density as a novel prognostic factor in early-stage invasive cervical cancer. Int. J. Cancer **95:** 29–33.
56. BIRNER, P., M. SCHINDL, A. OBERMAIR, *et al.* 2000. Lymphatic microvessel density in epithelial ovarian cancer: its impact on prognosis. Anticancer Res. **20:** 2981–2985.
57. DE WAAL, *et al.* 1997.
58. CLARIJS, *et al.* 2001.
59. CARREIRA, C.M., S.M. NASSER, E. DI TOMASO, *et al.* 2001. LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. Cancer Res. **61:** 8079–8084.

Molecular characterization of lymphatic endothelial cells

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Abbreviations: EC, endothelial cell; LEC, lymphatic EC; BEC, blood EC; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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Abstract

The lymphatic microvasculature is uniquely adapted for the continuous removal of interstitial fluid and proteins, and is an important entrypoint for leukocytes and tumor cells. Specialized functions of lymphatics suggest differences in the molecular composition of the lymphatic and blood vascular endothelium. However, the extent to which the two cell types differ is still unclear and few molecules that are truly specific to lymphatic endothelial cells have been identified to date. We have isolated primary lymphatic and blood microvascular endothelial cells from human skin by immunoselection with the lymphatic marker LYVE-1 and demonstrate that the two cell lineages express distinct sets of vascular markers and respond differently to growth factors and extracellular matrix. Comparative microarray analysis of gene expression profiles revealed a number of unique molecular properties which distinguish lymphatic and blood vascular endothelium. The molecular profile of lymphatic endothelium appears to reflect characteristic functional and structural features of the lymphatic capillaries. Classification of the differentially expressed genes into functional groups revealed particularly high levels of genes implicated in protein sorting and trafficking, indicating a more active role of lymphatic endothelium in uptake and transport of molecules than previously anticipated. The identification of a large number of genes selectively expressed by lymphatic endothelium should facilitate the discovery of novel lymphatic vessel markers and provide a basis for the analysis of the molecular mechanisms accounting for the characteristic functions of lymphatic capillaries.

Introduction

The lymphatic and blood vascular systems serve distinct yet complementary functions to maintain tissue homeostasis. The lymphatic system returns fluid and macromolecules from the tissues back to the blood circulation and thus plays a vital role in the regulation of fluid, protein and pressure equilibrium in tissues (1, 2). The lymphatic vessels also play an important role in the immune response by directing antigen-presenting cells from tissues to the lymph nodes (3).

Lymphatic capillaries are responsible for the uptake of the components from the interstitium. While endothelial cells of lymphatic capillaries have many properties in common with the endothelium of blood vessels, they also have distinct structural characteristics reflecting their specific functions (4-7). Lymphatic capillaries lack mural cells and are characterized by an incomplete or absent basement membrane. Lymphatic endothelium typically contains numerous invaginations and cytoplasmic vesicles as well as characteristic overlapping intercellular junctions. While the junctions in blood vessels connect adjacent endothelial cells over entire cell boundaries, in lymphatics the junctions are generally more sparse. Finally, one of the most striking characteristics of the lymphatic capillary is its integration within the interstitium; lymphatic endothelial cells are connected to the extracellular matrix by fine strands of elastic fibers, i.e. anchoring filaments (5, 8-10).

The unique structural and functional characteristics of lymphatic capillaries suggest significant differences between the lymphatic and blood microvasculature at the molecular level. However, very few differentially expressed molecules have been identified to date, and most of these are either expressed at lower levels or absent from lymphatics (11). Recently, several positive markers of lymphatic vessels have been identified. These include VEGFR-3, the tyrosine kinase receptor for vascular endothelial growth factor (VEGF)-C and VEGF-D (12); podoplanin, a glomerular podocyte membrane mucoprotein (13, 14); Prox-1, the homeobox gene product that is involved in developmental regulation of the lymphatic system (15) and a hyaluronan receptor LYVE-1 (16, 17). Still, better discrimination between the two types of capillaries is crucial for addressing questions regarding the biology and pathology of the lymphatic system.

In the present study we demonstrate for the first time the characteristic gene expression profile of human lymphatic microvascular endothelial cells. The identification of distinct molecular characteristics of lymphatics should provide insight into the molecular basis of lymphatic vessel function and help identify novel lymphatic vessel markers.

Materials and Methods

Isolation of Lymphatic and Blood Microvascular Endothelial Cells. Primary cultures consisting of a mixture of dermal cells were established from human neonatal foreskins according to a standard protocol (18). Cells were cultured on collagen-coated dishes in endothelial cell basal medium (Clonetics, Walkersville, MD) with 20% FBS and supplements, as described (19). Magnetic beads (Dynal, Lake Success, NY) were used for immunomagnetic purification of cells, according to the manufacturers instructions. Rabbit IgG-conjugated Dynabeads were coated with the anti-human LYVE-1 antibody

(16) and added to confluent primary cultures. Cells were incubated with beads for 15 min at 4°C, washed and trypsinized as described (19). LECs attached to beads were separated with a magnetic particle concentrator and plated. Cells in the supernatant were repeatedly exposed to the magnet to ensure removal of any remaining LECs bound to beads, and BECs were subsequently purified by incubating cells in suspension with the CD31-conjugated beads. The second immunopurification step was performed at passage 2. LECs were first depleted of CD34+ cells, and then purified using CD31-coated beads. BECs were depleted of any remaining LYVE-1+ cells and purified using CD34-coated beads. Beads were released from the cells with a DNase according to the manufacturers instructions (Dynal).

Immunofluorescent Staining. Cryosections of human foreskin tissue (6 or 50 μm thick) were stained as previously described (20), using antibodies to human LYVE-1 (16) (1/300), PAL-E (1/50; Caltag Laboratories, Burlingame, CA), CD31 (1/50; Dako, Carpinteria, CA), CD34 (1/50, Pharmingen, San Diego, CA) or smooth muscle α-actin (1/100; Dako) and corresponding secondary antibodies labeled with AlexaFluor488 or AlexaFluor594 (Molecular Probes, Eugene, Oregon). Cells were grown on coated tissue culture slides (ICN Biomedicals, Costa Mesa, CA) and fixed for 10 min in acetone before staining. Specimens were examined using a Nikon E-600 microscope and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, Michigan).

Cloning, Expression, and Purification of Recombinant Human VEGF-C. cDNA encoding mature VEGF-C (21, 22) was cloned from human umbilical vein endothelial cells (HUVECs). Total RNA was isolated using RNeasy kit (Qiagen, Chatsworth, CA), cDNA generated using SuperScript RT (Invitrogen, Carlsbad, CA) and human VEGF-C amplified by PCR (nt 657-995; GenBank accession number X94216). The purified PCR product was sub-cloned into EcoR I/BamH I-digested pSecTag2B expression vector containing C-terminal 6xHis tag and c-myc epitope (Invitrogen). The sequence-verified pSecTag2B/VEGF-C ΔNΔC construct was transfected into 293T cells using Fugene 6 Transfection Reagent (Roche, Palo Alto, CA) and stably transfected cells were selected in growth medium (DMEM with 10% FBS) containing 100 μg/ml Zeocin. Conditioned medium was collected after 48 hr incubation and concentrated 10-fold using Centriplus-10 filtration devices (Amicon, Beverly, MA). VEGF-C was purified using Ni-NTA Agarose (Qiagen), according to the manufacturers protocol. Concentration of the purified protein was determined with the BioRad assay (BioRad, Hercules, CA) and VEGF-C was analyzed by Western Analysis using antibodies to c-myc (Invitrogen) or VEGF-C (R&D Systems, Minneapolis, MN).

Collagen Gel Assay. The ability of LECs and BECs to form capillary-like structures in vitro was assessed in a collagen gel "sandwich" assay as previously described (23). ECs were seeded onto three-dimensional collagen gels at 2 x 10^4 cells/cm^2, and allowed to attach. After 90-120 minutes medium was aspirated and the cells were overlaid with a second layer of collagen. Cultures were treated with 10 ng/ml rhFGF-2 (kindly provided by Dr. P. Sarmientos, Farmitalia Carlo Erba, Milan, Italy), 100 ng/ml rhVEGF (PeproTech Inc, Rocky Hill, NJ) or 100 ng/ml rhVEGF-C. 48 hours later, cells were

analyzed by phase contrast microscopy using a Nikon Diaphot TMD microscope. The total length of cell cords in each 1.0 x 1.4 mm field was measured. Results were expressed in μm as the mean total cell cord length ± sem per field, from at least 15 measured fields per condition. Data were pooled from 4 experiments.

Northern and Western Analyses. Northern and Western analyses were performed as described (24), using confluent cells at passages 4 to 6. The LYVE-1 probe used for Northern analysis was a nt 90-1092 human LYVE-1 cDNA fragment (GenBank accession number AF118108) and was generated by RT-PCR from total RNA isolated from LECs using RNeasy kit (Qiagen). A single-tube RT-PCR was performed as indicated by the manufacturer (Stratagene, La Jolla, CA). A human β-actin cDNA probe (Clontech, Palo Alto, CA) was used as a control for equal RNA loading. Western analyses were performed using antibodies against human CD31, VEGFR-2, VEGFR-3 (all from Santa Cruz Biotechnology, Santa Cruz, CA or R&D Systems), plakophilin 2 (Research Diagnostic, Flanders, NJ), or LYVE-1 (16).

Affymetrix Microarray Analysis. Total RNA was isolated from LECs and BECs at passage 4 and DNA removed with the DNase kit (Qiagen). Human GeneChips (HG-U95Av2) were purchased from Affymetrix (Santa Clara, CA). Array HG-U95Av2 is comprised of ~12,000 sequences, most of which are previously characterized full-length genes; each gene is represented by ~16 nonoverlapping oligonucleotide probes (25-mers). cDNA synthesis, hybridization, and signal intensity normalization were carried out at the Affymetrix facility of the Brigham and Women's Hospital (Boston, MA). Data indicating presence or absence of gene expression (presence/absence call, determined by Affymetrix) were sorted, compared and statistically analyzed using Spotfire software (Somerville, MA). Sequences whose presence call was designated A in both cell types or M in either cell type were filtered out. Genes were considered selectively expressed when present in one but absent in the other cell type; fold change in this case designates the level of gene expression above an arbitrary treshold. Genes were considered differentially expressed when present in both cell types, with at least 2-fold difference in signal intensity; fold change in this case indicates the relative difference in signal intensity for the gene between the two cell types. To search for the sequences encoding LYVE-1, Prox-1 and podoplanin (GenBank accession numbers AF118108, U44060 and U96449 respectively) among ESTs, because they were not present among known genes, we developed a custom algorithm that translates query sequences into all 6 reading frames and compares them with the 6-reading frame translation of the target-sequences (25). Genes were designated according to the annotations from Affymetrix and the Genecards databases (http://www.dkfz-heidelberg.de/GeneCards). Genecards were used to identify gene function and the genes were classified into functional categories following the Gene Ontology consortium guidelines (http://www.geneontology.org) (26).

Results

Distinct expression patterns of vascular markers in lymphatic and blood vasculature. LYVE-1 is a hyaluronan receptor selectively expressed in lymphatic vessels in most tissues (16, 27), with the exception of spleen and liver where it is expressed also

by specialized sinusoids (16, 28). In human skin LYVE-1 unequivocally distinguishes lymphatic from blood microvascular endothelium, as assessed by immunostaining using the anti-LYVE-1 antibody in combination with several antibodies against blood vascular endothelial antigens (Fig. 1). Expression of LYVE-1 was restricted to a subset of dermal vessels lacking expression of PAL-E, a specific blood vascular marker. In contrast, CD31 was detected in all vessels, although expression levels were lower in the LYVE-1 positive endothelium (Fig. 1*B-D*). Blood capillaries also stained strongly for CD34, which was completely absent from LYVE-1 positive vessels (Fig. 1*E* and *F*). Another important distinction between blood and lymphatic microvasculature is the lack of mural cells around lymphatics (4-6). Accordingly, expression of smooth muscle α-actin, a marker of mural cells, was restricted to blood capillaries and was not detected in association with LYVE-1 positive vessels (Fig. 1*G* and *H*). These results demonstrate differences in vascular marker expression between lymphatic and blood microvasculature and confirm the selectivity of LYVE-1 for lymphatics in the skin.

Isolation of lymphatic and blood microvascular endothelial cells. Pure populations of microvascular LECs and BECs were isolated from human neonatal foreskins by immunomagnetic purification using a combination of vascular markers LYVE-1, CD31 and CD34. First, primary cultures consisting of a mixture of dermal microvascular endothelial cells, fibroblasts and some epidermal keratinocytes were established according to a standard protocol (18). Next, LECs were purified using magnetic beads coupled to a LYVE-1 antibody. Within primary cultures, LYVE-1 positive cells formed clusters that were segregated from the LYVE-1 negative endothelium (Fig. 2*A* and *B*). After the second purification step with the CD31 antibody (Fig. 2C), any remaining BECs were removed with the CD34 antibody. Immunofluorescent staining of LECs in culture demonstrated that all cells expressed CD31 at cell junctions (Fig. 2*D*). Corresponding BECs purified from the same pool of endothelial cells were defined as LYVE-1-/CD31+/CD34+ cells. LECs and BECs exhibited similar morphology as monolayer cultures under standard growth conditions (Fig. 2*E* and *F*), and were propagated for at least 8 passages without altering their characteristics. The procedure results in high yields; approximately 10^9 LECs and BECs can be obtained from a single foreskin by passage 5.

Selective effects of VEGF-C on cultured lymphatic endothelium. The ability of collagen type I and growth factors to induce capillary-like morphogenesis in LECs and BECs was assessed in a collagen gel "sandwich" assay (23). Within 48 hours of exposure of the apical cell surface to collagen type I, the majority of BECs and LECs had undergone cell death. Interestingly, this effect was more prominent in BECs than in LECs (Fig. 3*A* and *B*). LECs showed not only increased survival rates but also demonstrated the ability to form tubes without exogenously added growth factors. Addition of FGF-2 had no effect on survival of either cell type (Fig. 3*C* and *D*). VEGF, however, was a potent survival factor for both blood and lymphatic vascular endothelial cells and promoted the formation of an extensive network of tubes (Fig. 3*E* and *F*). In contrast, VEGF-C selectively induced survival and tube formation of LECs (Fig. 3*G* and *H*; Fig. 4). The effect of VEGF-C on LECs was comparable to that observed with VEGF treatment. The

differential responsiveness of the two cell lineages to the extracellular matrix and to VEGF-C indicates that LECs and BECs retain their distinct phenotypes in culture.

Differential expression of specific markers in cultured lymphatic and blood vessel ECs. LECs and BECs were analyzed for expression of several lineage-specific genes by Western and/or Northern analysis (Fig. 5). LYVE-1 was selectively expressed in LECs, both at RNA and protein levels. Both cell lineages maintained expression of CD31 in culture, as determined by Western analysis (Fig. 5) and immunostaining (Fig. 2). Expression of CD31 was less pronounced in cultured LECs, recapitulating the expression pattern observed in vivo. Likewise, lower amounts of VEGFR-2 protein were detected in LECs than in BECs, whereas VEGFR-3 protein was predominantly expressed by LECs. Plakophilin 2, desmosomal protein found in non-classical adherens junctions characteristic of lymphatic vessels (29, 30), was detected mainly in LECs. These results demonstrate that LECs and BECs stably maintain distinct patterns of gene expression in culture. Microarray analysis confirmed expression of CD31 and plakophilin 2 by LECs (Table 2). However, in contrast to the protein data, expression levels of VEGFR-2 and VEGFR-3 RNA were comparable between the two cell types, suggesting that differences in the amounts of the respective proteins result from posttranscriptional regulation. Sequences for lymphatic markers LYVE-1, podoplanin or Prox-1 were not present on the GeneChip among the known genes or ESTs, as determined by the custom algorithm (see methods).

Molecular profile of lymphatic vascular endothelial cells. To further characterize differences between lymphatic and blood endothelium we carried out microarray analysis. Of the 12626 genes represented on a GeneChip, 5789 were expressed in one or other cell type (45%). Among the expressed sequences, 33% were differentially expressed. Of all genes expressed, 20% were either selectively present or significantly upregulated in LECs, indicating notable quantitative as well as qualitative differences between the two cell lineages (Fig. 6).

To investigate whether certain classes of genes were preferentially represented by the lymphatic endothelium, the differentially expressed genes were classified based on their function following the Gene Ontology consortium guidelines (26). The categories that were most highly represented in LECs comprised molecules involved in protein transport, secretion and metabolism (Table I, supplementary data available online). Synaptogyrin 3, the gene expressed at the highest level within this group, is a member of the family of proteins abundantly present in synaptic vesicles, with presumable function in exocytosis (31, 32). LECs selectively expressed high levels of various genes encoding proteins of the SNARE family such as syntaxins 1a, 5, 11 and 16, YKT6 protein and VAMPs that play a central role in vesicular trafficking (32-34). Syntaxins are transmembrane proteins that regulate fusion of transport vesicles with target membranes. YKT6 and syntaxin 5, present on the vesicle and the target membrane, specifically interact to form complexes that catalyze lipid bilayer fusion (35). Besides SNAREs, LECs expressed transcripts encoding distinct members of other protein families that control the specificity of vesicle fusion, such as rab GTPases, sec-related proteins and AAA ATPases, as well as various genes that regulate vesicle docking (32, 34). Elevated

levels of transcripts for certain enzymes whose activity is required for protein translocation within the cell, were also typical for LECs.

Genes belonging to a number of other functional categories were differentially expressed. For example, LECs characteristically expressed several cell adhesion molecules that constitute adherens junctions, i.e. cadherin-13, plakophilin 2 and zona occludens 2 (ZO-2) (30, 36, 37), but did not express VCAM (vascular cell adhesion molecule) and N-cadherin, that were found selectively in BECs. As expected, BECs were characterized by prominent expression of several genes encoding the components of basement membrane, such as α1 type XV collagen, α3 laminin and nidogen. Several genes implicated in cell differentiation such as endothelial differentiation protein edg-1, ets-1, Id1 and Id2, were expressed in both cell types at the comparable levels. However, a transmembrane receptor implicated in endothelial differentiation, Notch4, was found expressed only by BECs. The differences were also found in the expression of chemokines and growth factors. Most prominently, BECs selectively expressed SDF-1 (stromal cell derived factor - 1), whereas RANTES, a chemokine for T-cells and monocytes, was primarily expressed by LECs. Several growth factors implicated in angiogenesis, such as bFGF, VEGF-B and TGFβ, were expressed in both lineages. Interestingly, LECs expressed high levels of VEGF and Ang2, whereas PlGF was predominantly expressed by BECs. The lists of differentially expressed genes are available online as supplementary data. Taken together, these results reveal significant differences in the molecular make-up of the lymphatic and blood microvascular endothelium and provide first insights into the molecular basis of the biological differences between the two cell lineages.

Discussion

The lymphatic microvasculature is uniquely adapted for the continuous removal of interstitial fluid and proteins, and is an important point of entry for leukocytes and tumor cells (1-3). The exact mechanisms by which lymphatic capillaries accomplish these tasks, however, remain to be defined. Specialized functions of lymphatics suggest differences in the molecular composition of lymphatic and blood vascular endothelium, the understanding of which should provide valuable insight into the molecular basis of lymphatic function. We have compared the gene expression profiles of isolated primary lymphatic and blood microvascular endothelial cells using commercially available microarrays, and demonstrate for the first time unique differences between the two cell types at the molecular level.

LECs and BECs were isolated from human skin by immunomagnetic separation using a combination of positive and negative markers. The purification strategy was devised based on the specific expression of LYVE-1 and CD34 in the lymphatic and blood vasculature of the skin, respectively. LECs were identified as LYVE-1+/CD31+/CD34- cells, whereas BECs were defined as LYVE-1-/CD31+/CD34+ cells. Both cell lineages retained this characteristic expression pattern of markers in culture, as demonstrated by Northern and Western analysis. Moreover, the lymphatic vessel growth factor VEGF-C selectively induced tube formation of LECs but not BECs in an in vitro angiogenesis assay. In contrast, VEGF promoted survival and tube formation of both cell types, suggesting that it might play a role in the regulation of lymphatic vessel survival

and/or formation in vivo. In agreement with the results of these functional studies, both cell lineages expressed VEGFR-2, whereas VEGFR-3 was predominantly expressed in LECs. Interestingly, in the absence of exogenous growth factors LECs incorporated into collagen type I scaffolds exhibited a significantly higher survival rate than BECs. This difference in response to collagen type I may reflect differences in the type of the extracellular matrix that each vessel type is exposed to in its natural environment. Blood vascular endothelium is in immediate contact with components of the basal lamina, whereas in lymphatic capillaries basal lamina is largely absent and LECs form an intimate association with adjacent interstitial tissue (5, 9). In fact, one of the features that discriminate lymphatic capillaries from blood capillaries at the ultrastructural level are direct connections of LECs to the interstitial collagens by anchoring filaments (5, 9). Taken together, distinct expression patterns of vascular markers by cultured LECs and BECs and their differential responsiveness to the extracellular matrix and VEGF-C indicate that LECs and BECs represent distinct cell lineages which retain their differentiated phenotypes in culture.

Recently, the feasibility of isolating LECs using two different lymphatic markers, podoplanin and VEGFR-3, has been reported (38, 39). In agreement with our results, these studies demonstrated that LECs maintained expression of their characteristic markers in culture. However, LECs isolated by the three different methods showed slightly different expression of some of the vascular markers examined, which may be due to the different isolation strategies selecting for distinct subpopulations of lymphatic endothelial cells. Alternatively, the reason may be a different source of tissues employed, i.e. adult vs. neonatal skin. LECs isolated from commercially available mixed cultures of endothelial cells by employing VEGFR-3 antibodies (38) may be partly contaminated with BECs, since VEGFR-3 can be expressed also by blood vascular endothelium (40).

Comparative analysis of gene expression profiles revealed significant differences in the molecular signatures of LECs and BECs. The molecular profile of LECs indeed appears to reflect the characteristic functional and structural features of the lymphatic microvascular endothelium. Classification of the differentially expressed genes into functional groups revealed that LECs express remarkably high levels of genes implicated in protein metabolism, sorting and trafficking. Particularly highly represented were genes encoding proteins that control specificity of vesicle targeting and fusion, such as proteins of the SNARE family, rab GTPases, AAA ATPases and sec-related proteins (32, 34), indicating pronounced vesicular transport in LECs. Of interest, one of the typical features of lymphatic endothelial ultrastructure is the presence of membrane invaginations and cytoplasmic vesicles (10, 41, 42), whose functional significance has not been established. Intercellular clefts are considered to be a major passageway for fluid and proteins into the lymphatics, the entry of which is driven by pressure gradients across the endothelial wall (43). Some early studies, however, demonstrated the presence of interstitially injected molecular tracers within intracellular vesicles of lymphatic endothelial cells (10, 41, 42). In agreement with these findings, our results strongly suggest that in addition to intercellular transport, transendothelial pathways may also be utilized as a mechanism for the entry of molecules into lymphatics. This raises an interesting possibility that lymphatics may have the capacity to selectively remove molecules from the interstitium and therefore actively control the composition of lymph and interstitial fluid.

Expression of several genes encoding proteins implicated in transport of solutes further suggests an active role of LECs in regulating interstitial homeostasis. The potassium/chloride cotransporter KCC1 for example, plays an important role in the control of extracellular fluid volume as well as in the control of membrane potential (44). Lymphatic endothelium is characterized by a high density of anionic sites on cell membranes, particularly along intercellular junctions, which have been suggested to facilitate movement of small solutes and molecules into the lymphatic lumen (45). Hence, the expression of specific ion transporters in lymphatic endothelium may directly or indirectly regulate transport of solutes and fluid into the vessel.

We have identified many other genes whose expression appears to be of relevance to the typical structure of lymphatic capillaries. Ang 2, for example, is implicated in destabilizing adhesion of mural cells to the endothelium of blood capillary (46). In lymphatics, constitutive expression of Ang2 by endothelial cells may account for the characteristic lack of pericytes in these vessels. Lymphatic capillaries are further distinguished by the specific organization of its intercellular junctions, including the typical presence of a special type of adherens junctions (29). We have identified several genes encoding proteins that constitute adherens junctions, such as plakophillin 2, H-cadherin and zona occludens 2 (ZO-2) (30, 36, 37). Finally, the lymphatic endothelial cytoskeleton is directly connected to the extracellular matrix by anchoring filaments composed mainly of elastin fiber microfibrils (9). Interestingly, an elastin microfibril protein (EMILIN) that is normally expressed in elastin-rich tissues (47) has been found selectively and abundantly expressed in LECs.

In conclusion, we reveal a number of unique properties by which lymphatic and blood microvascular endothelium can be distinguished. With the exception of the few newly identified positive lymphatic markers, most of the known vascular markers are present at lower levels or absent in lymphatics (11). Furthermore, none of the positive lymphatic markers known to date are exclusive for the lymphatic vasculature in all types of tissues, therefore, the necessity for the discovery of markers that would more reliably discriminate the two types of the vasculature in physiological as well as in pathological conditions remains. Identification of a large number of genes selectively expressed by LECs should facilitate the discovery of such markers. Finally, lymphatics have traditionally been assigned a passive role in the uptake of fluid, proteins and cells from the interstitium. Our results indicate a more active role of lymphatic endothelium than previously anticipated and should provide a basis for the future analysis of the molecular mechanisms that account for the characteristic functions of lymphatic capillaries.

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Figure legends

Fig. 1. Selective expression of vascular markers in human skin vasculature. (*a*) Double immunofluorescent staining for LYVE-1 (red) and a PAL-E, a marker of blood vessels (green), in 50 μm thick section of human foreskin. The stainings are mutually exclusive indicating high specificity of LYVE-1 antibody for lymphatic vessels. Note high lymphatic vessel density. (*b-d*) Fluorescent staining respectively for LYVE-1 (red), CD31 (green) or both together (merged), demonstrates that all LYVE-1 positive vessels are also CD31 positive. (*e,f*) Double-staining for CD34 (red) and PAL-E (green) revealed identical expression pattern in blood vessels (*e*), whereas LYVE-1 positive lymphatic vessels (green) do not express CD34 (*f*). (*g,h*) Smooth muscle α-actin (red) was colocalized with PAL-E (green) in blood vessels (*g*), but was absent from LYVE-1 positive vessels (*h*). Arrows point to the lymphatics, arrowheads to blood vessels. Dots indicate dermal-epidermal junction. Scale bar = 100 μm.

Fig. 2. Purification of lymphatic and blood microvascular endothelium. (*a*) Primary culture consisting mainly of endothelial cells, a few fibroblasts and keratinocytes, with LYVE-1-coated magnetic beads attached to the subpopulation of endothelial cells. (*b*) After the second purification step all cells are stained with the CD31 antibody at cell junctions, indicating a pure endothelial population. (*c,d*) Confluent layers of lymphatic (*c*) and blood vascular (*d*) endothelial cells at passage 7. Scale bar = 25 μm.

Fig. 3. Selective effects of VEGF-C on survival and tube formation by LECs in a collagen gel. Cells either received no treatment (*a,b*) or were exposed to exogenous FGF-2 (*c,d*), VEGF (*e,f*) or VEGF-C (*g,h*). Scale bar = 25 μm.

Fig. 4. Quantitative assessment of LEC and BEC network formation in the collagen gel. Total length of cell cords formed after 48 hr was measured. Data are expressed as total cord length (in μm $\pm$ sem) per field from at least 15 fields. Data are pooled from 4 experiments. The unpaired Student's t-test was used for statistical analyses. LEC control vs. FGF, $p=0.5$; vs. VEGF, $p<0.001$; vs. VEGF-C, $p<0.001$; BEC control vs. FGF, $p=0.5$; vs. VEGF, $p<0.001$; vs. VEGF-C, $p<0.05$.

Fig. 5. Differential expression of vascular markers by cultured LECs and BECs. (*a*) Western and Northern analyses of LYVE-1 expression. Two major bands of LYVE-1 protein (about 70 and 200kDa) and RNA (2.0 and 2.6 kb) were expressed in LECs. Hybridization with a β-actin cDNA probe was performed as a loading control for the Northern. For Westerns, equal amounts of proteins were blotted. (*b*) Western analyses of CD31, VEGFR-2, VEGFR-3 and plakophilin expression. Positions of molecular weight markers are shown in kDa.

Fig. 6. Quantitative assessment of differential gene expression.

References

1. Rusznyak, I., Foldi, M. & Szabo, G. (1967) *Lymphatics and lymph circulation* (Pergamon Press, Oxford).

2. Swartz, A. & Skobe, M. (2001) *Microsc Res Tech* **55**, 92-99.

3. Yoffey, J. M. & Courtice, F. C. (1970) *Lymphatics, lymph and the lymphomyeloid complex* (Academic Press, London).

4. Casley-Smith, J. R. & Florey, H. W. (1961) *Quart J Exp Physiol* **46**, 101.

5. Leak, L. V. (1970) *Microvasc. Res.* **2**, 361-391.

6. Daroczy, J. (1988) *The dermal lymphatic capillaries* (Springer-Verlag, Berlin, Heidelberg).

7. Skobe, M. & Detmar, M. (2000) *J Invest Dermatol Symp Proceed* 5(1):14-19.

8. Pullinger, B. D. & Florey, H. W. (1935) *Br J Exp Pathol* **16**, 49.

9. Gerli, R., Ibba, L. & Fruschelli, C. (1990) *Anat. Embryol.* **181**, 281-286.

10. Leak, L. V. (1976) *Fed Proc* **35**, 1863-71.

11. Sleeman, J. P., Krishnan, J., Kirkin, V. & Baumann, P. (2001) *Microsc. Res. Tech.* **55**, 61-69.

12. Kaipainen, A., Korhonen, J., Mustonen, T., van, H. V., Fang, G. H., Dumont, D., Breitman, M. & Alitalo, K. (1995) *Proc Natl Acad Sci U S A* **92**, 3566-70.

13. Breiteneder-Geleff, S., Soleiman, A., Kowalski, H., Horvat, R., Amann, G., Kriehuber, E., Diem, K., Weninger, W., Tschachler, E., Alitalo, K. *et al.* (1999) *Am J Pathol* **154**, 385-94.

14. Weninger, W., Partanen, T. A., Breiteneder-Geleff, S., Mayer, C., Kowalski, H., Mildner, M., Pammer, J., Sturzl, M., Kerjaschki, D., Alitalo, K. *et al.* (1999) *Lab Invest* **79**, 243-51.

15. Wigle, J. T. & Oliver, G. (1999) *Cell* **98**, 769-78.

16. Banerji, S., Ni, J., Wang, S. X., Clasper, S., Su, J., Tammi, R., Jones, M. & Jackson, D. G. (1999) *J Cell Biol* **144**, 789-801.

17. Jackson, D. G., Prevo, R., Clasper, S. & Banerji, S. (2001) *Trends Immunol* **22**, 317-21.

18. Davison, P. M., Bensch, K. & Karasek, M. A. (1980) *J. Invest. Dermatol.* **75**, 316-321.

19. Richard, L., Velasco, P. & Detmar, M. (1998) *Exp. Cell Res.* **240**, 1-6.

20. Skobe, M., Hawighorst, T., Jackson, D. G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K. & Detmar, M. (2001) *Nat Med* **7**, 192-198.

21. Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N. & Alitalo, K. (1996) *Embo J* **15**, 290-298.

22. Joukov, V., Kumar, V., Sorsa, T., Arighi, E., Weich, H., Saksela, O. & Alitalo, K. (1998) *J. Biol. Chem.* **273**, 6599-6602.

23. Montesano, R., Orci, L. & Vassalli, P. (1983) *J Cell Biol* **97**, 1648-52.

24. Skobe, M., Hamberg, L. M., Hawighorst, T., Schirner, M., Wolf, G. L., Alitalo, K. & Detmar, M. (2001) *Am. J. Pathol.* **159**, 893-903.

25. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997) *Nucleic Acids Res.* **25**, 3389-3402.

26. Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T. *et al.* (2000) *Nat Genet* **25**, 25-9.

27. Prevo, R., Banerji, S., Ferguson, D. J., Clasper, S. & Jackson, D. G. (2001) *J Biol Chem* **276**, 19420-30.

28. Carreira, C. M., Nasser, S. M., di Tomaso, E., Padera, T. P., Boucher, Y., Tomarev, S. I. & Jain, R. K. (2001) *Cancer Res* **61**, 8079-84.

29. Schmelz, M., Moll, R., Kuhn, C. & Franke, W. W. (1994) *Differentiation* **57**, 97-117.

30. Mertens, C., Kuhn, C. & Franke, W. W. (1996) *J. Cell Biol.* **135**, 1009-1025.

31. Sugita, S., Janz, R. & Sudhof, T. C. (1999) *J Biol Chem* **274**, 18893-901.

32. Hay, J. C. (2001) *Exp Cell Res* **271**, 10-21.

33. Nelson, W. J. & Yeaman, C. (2001) *Trends Cell Biol.* **11**, 483-486.

34. Mellman, I. & Warren, G. (2000) *Cell* **100**, 99-112.

35. Zhang, T. & Hong, W. (2001) *J Biol Chem* **276**, 27480-7.

36. Lee, S. W. (1996) *Nat. Med.* **2**, 776-782.

37. Avila-Flores, A., Rendon-Huerta, E., Moreno, J., Islas, S., Betanzos, A., Robles-Flores, M. & Gonzalez-Mariscal, L. (2001) *Biochem. J.* **360**, 295-304.

38. Makinen, T., Veikkola, T., Mustjoki, S., Karpanen, T., Catimel, B., Nice, E. C., Wise, L., Mercer, A., Kowalski, H., Kerjaschki, D. *et al.* (2001) *EMBO J.* **20**, 4762-4773.

39. Kriehuber, E., Breiteneder-Geleff, S., Groeger, M., Soleiman, A., Schoppmann, S. F., Stingl, G., Kerjaschki, D. & Maurer, D. (2001) *J. Exp. Med.* **194**, 797-808.

40. Partanen, T. A., Alitalo, K. & Miettinen, M. (1999) *Cancer* **86**, 2406-12.

41. Dobbins, W. O., III & Rollins, E. L. (1970) *J. Ultrastruct. Res.* **33**, 29-59.

42. Leak, L. V. (1972) *J. Ultrastruct. Res.* **39**, 24-42.

43. Schmid-Schönbein, G. W. (1990) *Physiol. Rev.* **70**, 987-1028.

44. Delpire, E. & Mount, D. B. (2002) *Annu. Rev. Physiol* **64**, 803-843.

45. Leak, L. V. (1986) *Microvasc. Res.* **31**, 18-30.

46. Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N. *et al.* (1997) *Science* **277**, 55-60.

47. Doliana, R., Mongiat, M., Bucciotti, F., Giacomello, E., Deutzmann, R., Volpin, D., Bressan, G. M. & Colombatti, A. (1999) *J Biol Chem* **274**, 16773-81.

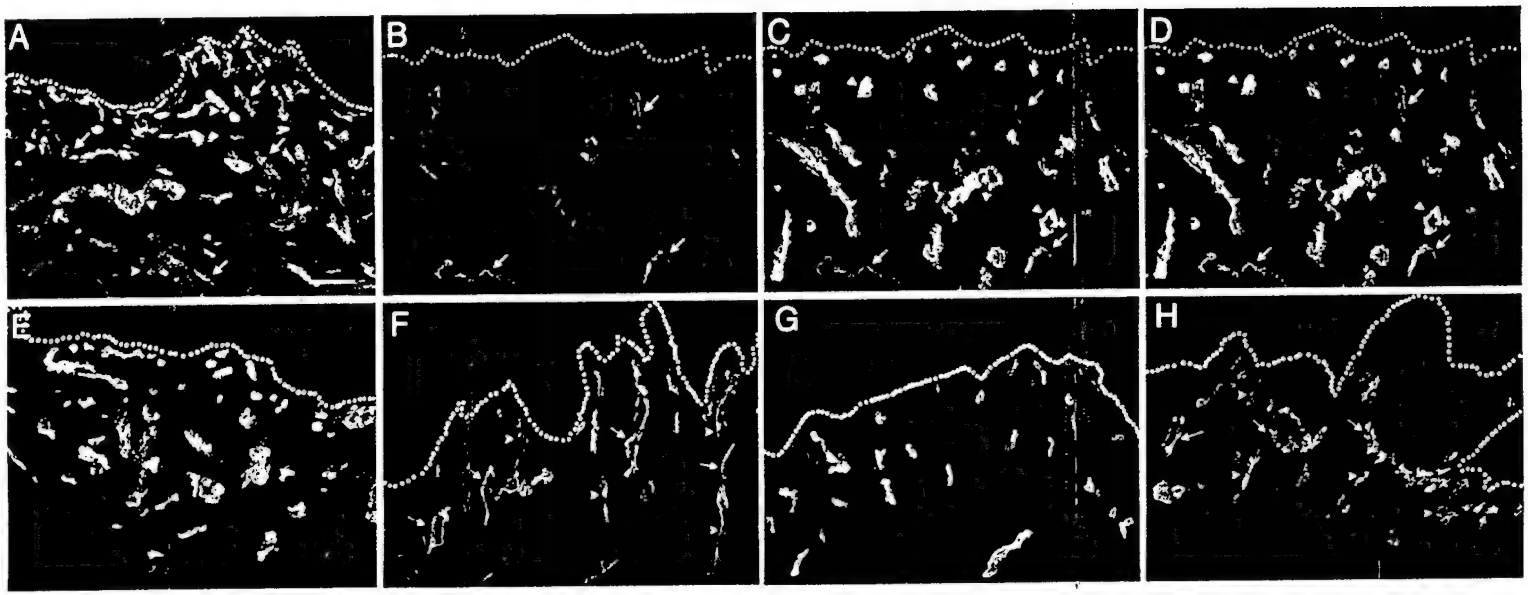


Figure 1

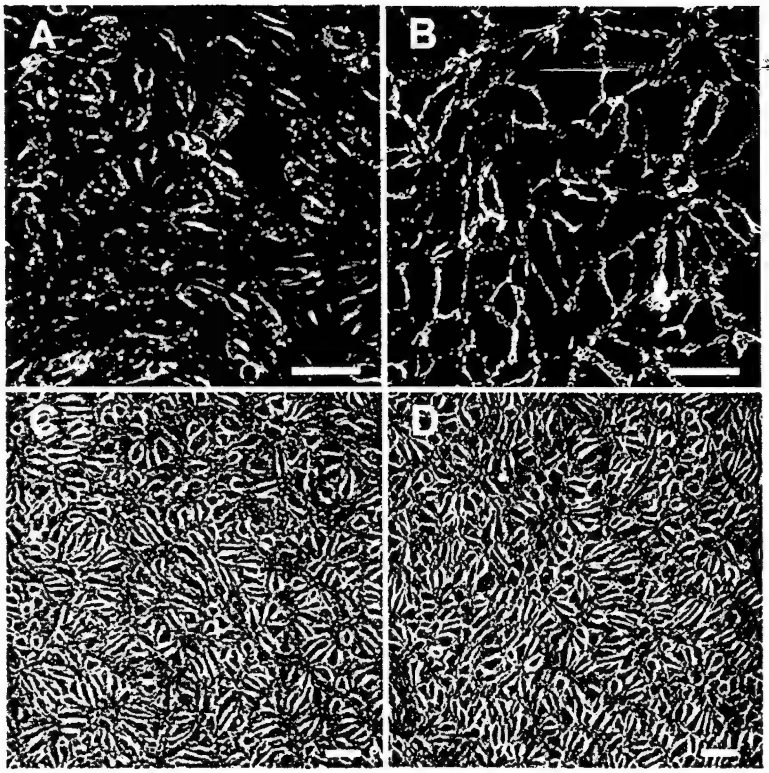


Figure 2

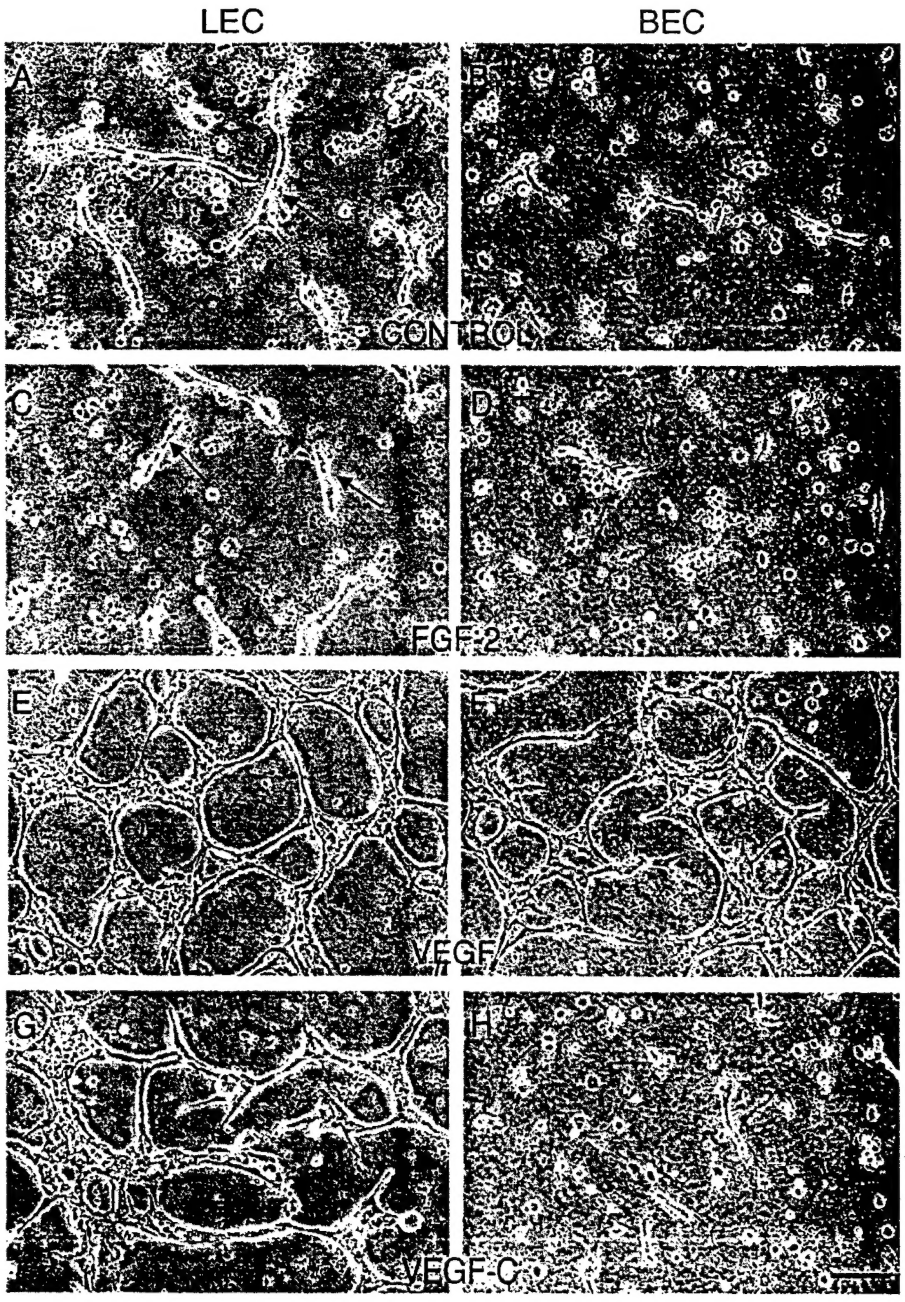
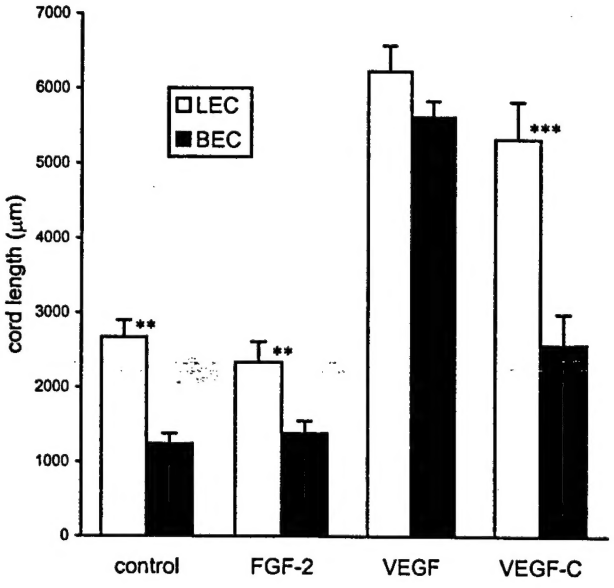


Figure 3


7000
6000
5000
4000
3000
2000
1000
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cord length (µm)
LEC
BEC
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**
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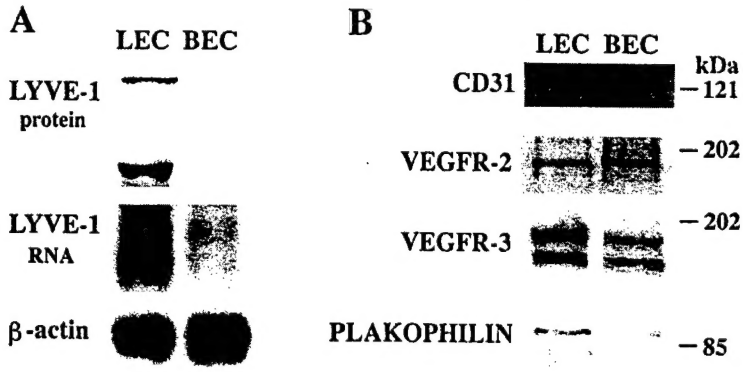
control
FGF-2
VEGF
VEGF-C

Figure 4


A
LEC BEC
LYVE-1
protein
LYVE-1
RNA
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CD31
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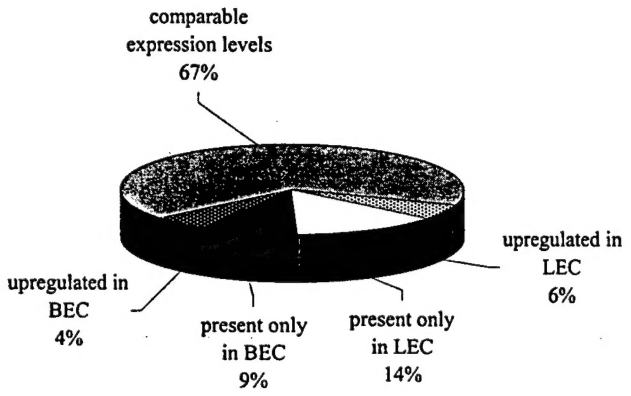
VEGFR-2
202
VEGFR-3
202
PLAKOPHILIN
85

Figure 5



Expression in LECs	Total genes	Known	Novel
specific	790	538	252
upregulated	324	233	91
downregulated	302	221	81

Figure 6